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ISOLATION AND CHARACTERISATION OF AN INHIBITOR
OF COMPLEMENT MEDIATED PREVENTION OF
IMMUNE PRECIPITATION.

BY

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B.Sc. M.Sc.

Thesis submitted for the degree of Ph.D. in
the faculty of Medicine, University of Glasgow,
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Abbreviations

BSA	Bovin serum albumin
C	Hill constant
Con.A	Concanavalin A
cpm	Counts per minutes
DESZ	Diethylaminoethyl cellulose
DEAE	Diethylaminoethyl cellulose
EA	Antibody-coated sheep erythrocytes
EACA	ϵ -amino-n-caproic acid
ELISA	Enzyme-linked immunosorbent assay
gp	Glycoprotein
gp60	Glycoprotein 60 (60 kD glycoprotein)
hrs	Hours
IC	Immune complex(es)
K	Affinity constant
kD	Kilodalton
MAC	Membrane attack complex
mins	Minutes
O.D	Optical density
PIP	Prevention of immune precipitation
PMSF	Phenyl methyl sulphonyl fluoride
r	Linear regression coefficient
RIA	Radioimmunoassay
RF	Rheumatoid factor
SBTI	Soya bean trypsin inhibitor
SD	Standard deviation

Abbreviations continued

SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
TEMED	N,N,N',N'-tetramethylethylenediamine
TFMS	Trifluoromethylsulphonic anhydride
Tween-20	Polyoxyethylene-sorbitan monolaurate
v/v	Volume for volume
w/v	Weight for volume

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PUBLICATIONS.

Some of the work presented in this thesis has been published in the following journals:

AHMED, A.E.E. & WHALEY, K. (1988). Purification of a plasma protein which inhibits complement mediated prevention of immune precipitation. Immunology, 64:45.

AHMED, A.E.E., BIRD, P., McKAY, I. & WHALEY, K. (1989). The protein that inhibits complement mediated prevention of immune precipitation is an Fc binding protein. Immunology, 66:20.

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SUMMARY

A normal plasma protein that inhibits complement-mediated prevention of immune precipitation (PIP) has been purified from normal human serum by three different methods. The first method was based on sequential affinity chromatography on IgG-Sepharose, protein A-Sepharose, and Con.A-Sepharose which resulted in the purification of a protein which was 60 kD on SDS-PAGE, 2.9S on sucrose density gradient centrifugation, 58 kD molecular weight on gel filtration and had a pre-albumin electrophoretic mobility. The second method which was a modification of the first method involving the use of protease inhibitors during the purification procedure, produced a protein which was 60 kD molecular weight on SDS-PAGE, 19 S on sucrose density gradient centrifugation, 1000 kD molecular weight by Sephacryl S-300 gel filtration and had a β_2/γ electrophoretic mobility. The third method did not involve an IgG-Sepharose affinity chromatography step but consisted of a combination of ion-exchange chromatography, gel filtration and heparin-Sepharose affinity chromatography with a final yield of 38% and 3360- fold purification. The final product of this purification procedure was a protein which shared the same characteristics with that prepared by method 2.

A monospecific antiserum was produced by immunising rabbits with the protein purified by the first method. When tested in double-diffusion in agarose gel, the antiserum gave a reaction of complete identity with all three protein

preparations. The present data suggest that the high molecular weight form of the protein is the polymer of the low molecular weight form.

The purified protein stained positively in the PAS reaction, was sensitive to neuraminidase and trifluoromethylsulphonic anhydride treatment, indicating that it is a glycoprotein. On account of its molecular weight on SDS-PAGE (60 kD), it has been called glycoprotein 60 (gp60).

An ELISA procedure was developed using the anti-gp60 antiserum. The assay enabled me to measure gp60 levels in serum and to study the behaviour of serum gp60 on gel-filtration chromatography and sucrose density gradient centrifugation. Serum gp60 was found to be mainly in the high molecular weight form (19 S, 1000 kD), although a small proportion was of low molecular weight (2.9 S, 58 kD). Thus serum gp60 exists mainly as the polymer with a minor proportion being present as the monomer.

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In binding studies, [I]-gp60 was shown to bind to IgG but not to the IgA or IgM isotypes. The binding was localised to the Fc piece of IgG, and the subclasses IgG1 and IgG3 bound gp60 more effectively than did IgG2 or IgG4. 125
Analysis of the data from the binding of [I]-gp60 to solid-phase BSA-IgG anti-BSA immune complexes (IC) showed that the affinity constant of gp60 for IgG was between 6.6×10^8 and 11.2×10^8 l/mol for the monomer and 2.3×10^9 and 5.1×10^9 l/mol, and a single class of binding site was present. Saturation of binding was achieved when one molecule of gp60 monomer was bound for every five molecules of IgG, or when one molecule of gp60 polymer was bound for every 24 molecules of IgG.

Further binding studies revealed that gp60 competed with C1q, IgM-RF and F(ab')₂ IgG-RF for binding to the Fc piece of IgG.

Purified gp60 produced dose-dependent inhibition of PIP and solubilisation. PIP was the more sensitive to the effect of gp60. The polymeric form of gp60 inhibited PIP and solubilisation more effectively than the monomer.

The mechanism of action of gp60 was studied by adding gp60 to normal serum and showing that it produced dose-dependent inhibition of IC-mediated activation of the classical pathway as shown by reduced formation of the C1s-C1 inhibitor complex, reduced consumption of C4a and C2 and reduced C4a and C3a generation. These data showed that gp60 prevents activation of the classical pathway by preventing C1 binding to IC. This leads to reduced C3 convertase formation and C3 cleavage so that the binding of C3b to IC is limited.

Levels of gp60 in rheumatoid arthritis sera correlated with their ability to inhibit PIP.

Gp60 which was present in normal serum was shown to regulate complement activation as the addition of Fab anti-gp60, but not normal rabbit IgG Fab, to sera resulted in 1) increased levels of haemolytic complement when EA(IgG) were used as targets, 2) increased complement activation by IC and 3) increased levels of PIP activity. It was concluded that in normal serum gp60 regulated complement activation.

The identity of gp60 is unknown. It was shown to be distinct from the proteins recognised by 57 antisera. So

far attempts at amino acid sequencing have failed, and cDNA expression libraries are being screened to identify positive cDNA clones which can be used for nucleotide sequencing.

CHAPTER 1
INTRODUCTION

The complement system plays a major role in host defence against infection and in the inflammatory process. Since it was discovered, there has been a great increase in knowledge which has led to a better understanding of its role in health and in the patho-physiological aspects of many diseases.

In this introduction, I shall briefly review the history of the complement system, nomenclature, biochemistry, reaction mechanisms, and the role of complement in the elimination of antigen-antibody complexes (IC).

1.1 HISTORICAL ASPECTS

During the last century, it was noted that human serum was capable of killing bacteria (Grohmann, 1884). This observation was later confirmed by Buchner (1889), who showed that fresh serum was bactericidal and this activity was heat-sensitive and lost on dialysis against distilled water. Later in the same year, Buchner also demonstrated the ability of serum to lyse erythrocytes from another species, a process now known as immune haemolysis. Therefore, at this time, it was concluded that the lytic activity of fresh serum was due to an enzymatic activity, which was named alexin.

In 1896 Bordet suggested that this bactericidal activity of fresh serum was due to two factors, one heat-stable, which was present only in immune serum (now known to be antibody), while the other, which was heat-sensitive, was present in both immune and non-immune sera. This heat-

labile factor was called alexin.

Since then, with the advances in medical sciences and technology, the complement system has been extensively characterized both biochemically and functionally. It is now understood that the system consists of more than 25 self-assembling proteins acting in concert.

Like the other mediator systems that participate in the inflammatory process, the coagulation, kinin-forming and fibrinolytic systems, the activity of the complement system is carefully controlled. At several steps in the sequence a zymogen is activated to its proteolytically active form which then activates another zymogen to form a new protease, which in turn acts on another protein. Thus the activation process continues (Ruddy, Gigli & Austin, 1972).

For many years, it was known that cytolysis was the only function of complement. Now there are many functions considered to be of equal or greater importance, particularly those involved in the mediation of the inflammatory response, and those involving complement receptors.

1.2 NOMENCLATURE

Each of the proteins involved in the classical and the terminal sequences is denoted by the letter C followed by a number e.g. C1, C4, C2, C3, C5, C6, C7, C8 and C9 (WHO, 1968). The alternative pathway components are termed factors and each is represented by a letter e.g. Factor B, Factor D and properdin (Factor P). These are usually abbreviated to B, D and P respectively (WHO 1981).

The control proteins are referred to by the abbreviated forms of their trivial names, e.g C1 inhibitor (C1-INH), C4 binding protein (C4BP) and recently C3b inactivator and B1H globulin have been termed factor I and H respectively.

The enzymatically active forms of the components have a bar over the symbols, e.g $\overline{C1}$, $\overline{C1s}$. Cleavage fragments are indicated by suffixed lower case letters e.g, C4a, C4b, C4c and C4d.

The polypeptide subunit chains of each component are suffixed with Greek letters, starting with α for the largest chain, then β and γ respectively e.g C4 α , C4 β and C4 γ . Throughout the text the abbreviated symbols for complement components will be used.

1.3 CHEMISTRY AND REACTION MECHANISMS

The complement system can be divided into four groups of proteins, the classical pathway, the alternative pathway, the terminal sequence and a group of regulatory proteins. An outline of the complement system is shown in Fig. 1.1

The proteins of the classical pathway [Table 1.1], and the alternative pathway [Table 1.2], are responsible for the generation of the enzymes which activate C3 and C5 by limited proteolysis, while the components of the terminal sequence [Table 1.3], are required for complement-mediated cytotoxicity. Regulatory proteins control the activation and turn-over of the complement system. The fluid-phase regulatory proteins are shown in Table 1.4.

As the subject of this thesis is a study of a plasma protein which inhibits classical pathway complement-mediated activity, the prevention of immune precipitation (PIP), the components and mechanisms of the classical pathway activation will be covered in greater depth than other aspects of complement activation.

Figure 1.1:

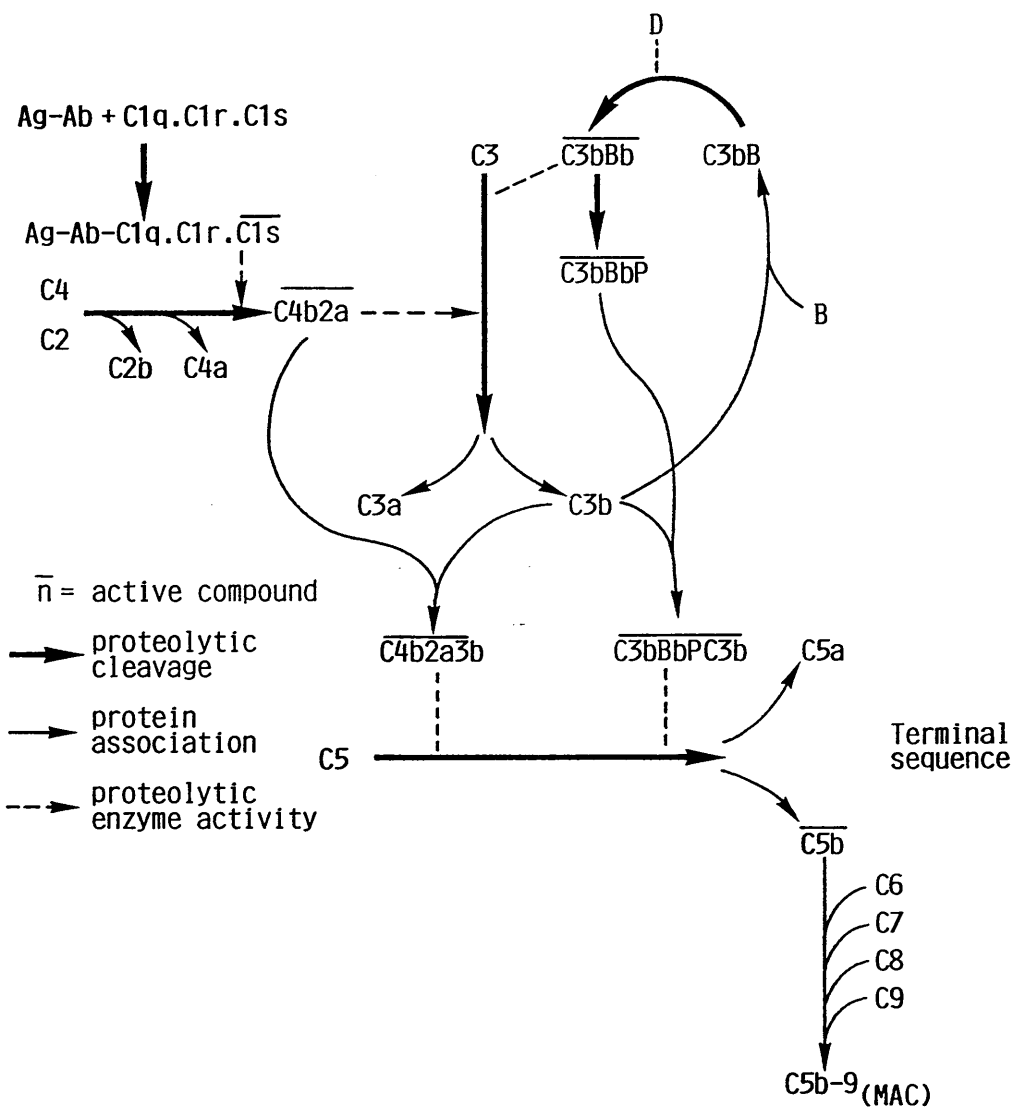
Schematic diagram for

complement activation pathways

COMPLEMENT ACTIVATION

CLASSICAL PATHWAY

ALTERNATIVE PATHWAY



1.4 CLASSICAL PATHWAY ACTIVATION

The classical pathway consists of three components, C1, C4 and C2 which upon activation, form the classical pathway C3 activating enzyme (C3 convertase) C4b2a, the formation of which is under the control of three regulatory proteins, C1-inhibitor, C4BP and factor I.

1.5 THE C1 MACROMOLECULE

The recognition unit of the classical pathway is the C1 macromolecule, which has a molecular weight of 750 kD. It is a metalloprotein complex consisting of three reversibly interacting subunits C1q, C1r and C1s (molar ratio 1:2:2 respectively), held together by calcium ions (Gigli et al, 1976). It is converted to its enzymatically active form by antigen-antibody complexes (IC) or other activators.

C1q is a large, collagen-like, structurally complex molecule which has a molecular weight of 410 kD (Calcott & Muller-Eberhard, 1972). It contains three types of polypeptide chain termed A, B and C. From amino acid sequence and electron microscopic observations (Reid & Porter, 1978), it was found that C1q consists of six A chains, six B chains, and six C chains, assembled to form triple helices.

A disulphide bond connects each pair of A and B chains within a single triple helix, and a second disulphide bond connects C chains in adjacent triple helices to form a pair of helices and heads. Three such pairs are then assembled to form the complete C1q molecule. The collagen-like

TABLE 1.1 CLASSICAL PATHWAY PROTEINS

Component	Molecular Weight kD	Serum Concentration µg/ml	Polypeptide Chain Structure	Cleavage Products	Biological Activity
Clq	410	70	18 (6x3)	-	Binds to antibody
Clr	85	110	1	-	Activates Cls
Cls	90	80	1	-	Activates C4 and C2
C4	204	430	3	C4a C4b	Anaphylatoxin C4b2a, C4b2a3b convertase
C2	100	20	1	C2a C2b	Enzymatic site of C4b2a Binds to C4b

TABLE 1.2 ALTERNATIVE PATHWAY PROTEINS

Component	Molecular Weight kD	Serum Concentration µg/ml	Polypeptide Chain Structure	Cleavage Products	Biological Activity
C3b	181	?	2	C3bi C3c C3dg	Ligand for CR3 ? Ligand for CR2
B	93	150	1	Ba Bb	Chemotactic Enzymatic site for C3bBb
D	25.5	2	1	-	Cleaves B
P	220	30	4	-	Stabilizes C3bBb

TABLE 1.3 TERMINAL SEQUENCE PROTEINS

Component	Molecular Weight kD	Serum Concentration µg/ml	Polypeptide Chain Structure	Cleavage Products	Biological Activity
C3	190	1300	2	C3a C3b	Anaphylatoxin Part of C5 convertase Part of C3bBb Ligand for CRI Solubilization of IC
C5	185	75	2	C5a C5b	Anaphylatoxin, Chemotactic Part of MAC
C6	128	60	1	-	Part of MAC
C7	121	60	1	-	Part of MAC
C8	153	80	3	-	Part of MAC
C9	79	50	1	-	Part of MAC

TABLE 1.4 FLUID-PHASE REGULATORY PROTEINS OF THE COMPLEMENT SYSTEM

Component	Molecular Weight kD	Serum Concentration µg/ml	Polypeptide Chain Structure	Cleavage Products	Biological Activity
C1 inhibitor	110	180	1	-	Serine protease inhibitor
C4BP	540	250	8	-	Co-factor for C4b degradation
I	90	50	2	-	Degrades C3b and C4b
H	150	300	1	-	Co-factor for C3b degradation
S.protein	88	150	1	-	MAC inhibition
Carboxy-peptidase N	300	-	8	-	Anaphylatoxin inactivation

sequence of amino acids begins close to the N-terminus and continues to about residue 88-90. The remaining 125-135 residues are folded to form the globular head of C1q.

The role of C1q in serving as the recognition unit of C1 mediated binding of the macromolecule to IC, and various C1 binding substances is well documented (Muller-Eberhard, 1975; Loos, 1983; Cooper, 1985).

Immunoglobulins exhibit specificity in their ability to bind C1q. The binding site on IgG lies within the CH2 domain, with IgG3 being the most efficient binder followed by IgG1 and IgG2. IgG4 does not bind C1q although its Fc piece possesses this function (Colomb & Porter, 1975; Isenman et al, 1975; Schumaker et al, 1976). IgM also binds to C1q through the CH3 domain, but IgA, IgD, and IgE isotypes do not appear to bind C1q (Ishizaka et al, 1967).

Monomeric fluid-phase IgG and IgM interact weakly with C1q⁶ with an affinity of $1-5 \times 10^6$ l/mol, but this affinity is increased when the immunoglobulin has been aggregated, or is part of an antigen-antibody complex (Wright et al, 1980).

The interaction between C1q and IgG is increased by lowering the ionic strength (Burton et al, 1980). Furthermore, the interaction appears to be highly conserved through evolution as human C1q reacts with IgG from different species (Alexander & Steiner, 1980).

C1r and C1s are very similar proteins, in terms of their amino acid sequence, size, carbohydrate content and function. Both are activated by limited proteolytic cleavage into two disulphide-linked peptides, with their active sites being located close to the C-terminus. In

their active forms each is a serine-protease type enzyme. This resemblance between C1r and C1s gave rise to the notion that they may arise by gene duplication (Arlaud & Gagnon, 1981). C1r exists in nature as a non-covalent dimer with an apparent molecular weight of 170 kD (Cooper, 1985). Its dimerization is independent of calcium ions, and it undergoes reversible pH-dependent dissociation into its monomeric form at pH 5 (Arlaud et al, 1980). C1r undergoes spontaneous fragmentation into 60 and 35 kD peptides when incubated at 37 °C in the presence of EDTA. This fragmentation results in C1r activation (Ziccardi & Cooper, 1976). The mechanism of C1r activation *in vivo* is not fully understood (Cooper, 1985; Schumaker, Zavodszky & Poon, 1987). However, it is thought that activation of C1r involves one monomeric subcomponent of the dimer cleaving the other. Activated C1r, ($\overline{\text{C1r}}$), then acts on natural substrate C1s (Valet & Cooper, 1974; Ziccardi & Cooper, 1976; Arlaud, Reboul & Colomb, 1977).

C1s is a single polypeptide chain of 87 kD, which undergoes calcium-dependent dimerization. Although C1s is very sensitive to proteolytic activation, it does not auto-activate. As with C1r, activated C1s ($\overline{\text{C1s}}$) is composed of two disulphide-linked polypeptide chains, the smaller of which possesses the serine esterase site which is responsible for cleaving C4 and C2.

Under physiological conditions, equimolar amounts of C1r and C1s undergo calcium-dependent association to form a flexible, linear tetramer, with the sequence C1s-C1r-C1r-C1s (Tschopp et al, 1980). The tetramer appears to be held

together through C1r-C1s contacts between the N-terminal interaction domains, whereas, the C-terminal catalytic domains of C1s appears to be located at the ends of the tetramer, and the catalytic domains of C1r are in contact at the centre of the sequence (Weiss et al, 1986).

1.6 THE STRUCTURE AND ACTIVATION OF C1

It is well known, that 70% of the C1 sub-components in serum are associated in the C1 complex (Ziccardi & Cooper, 1977). In the complexed form of C1, the C1s₂-C1r₂ tetramer is arranged around the collagenous arms of C1q. Final agreement on the precise configuration of the tetramer in the C1 macromolecule has yet to be achieved. However, one of the more popular models suggests that the tetramer is wrapped around the C1q cone in such a way that the catalytic domains of C1s and C1r are in contact with each other within the cone while the C1r-C1s interaction (contact) domains are outside the cone (Colomb, Arlaud & Viller, 1984; Weiss, Fauser & Engel, 1986; Schumaker, Zavodszky & Poon, 1987). The pro-enzyme C1 tends to auto-activate as shown by Ziccardi (1982) but in the absence of a C1-activator, this spontaneous activation is prevented by C1-inhibitor binding reversibly to the potentially active sites of the C1r and C1s tetramer. Upon binding to an activator, C1q undergoes a conformational change, which is transmitted along the collagen-like stalks to the C1r molecule which presumably also undergoes a conformational change. This exposes a catalytic site in C1r which cleaves the neighbouring C1r molecule. This auto-catalysis results in the generation of activated C1r (C1r) which will cleave and activate C1s. This process results in reduction of the association forces between the C1q and C1r₂-C1r₂, allowing the tetramer to unfold and to extend its C1s catalytic domains outside the C1q cone for interaction with C4 and C2 (Schumaker, Zavodszky & Poon, 1987).

Although the precise mechanism of C1 activation by immunoglobulins is not fully understood, there is even less information available on the mechanism of C1 activation by the wide variety of non-immunoglobulin substances including lipids, viruses, and parasites which are listed in Table 1.5. Indeed there are no common biochemical features shared between these substances and immunoglobulins.

Although C1 binding is a basic requirement for C1 activation (Tschopp, 1982) it is well documented that C1 binding to an acceptor surface does not always lead to its activation (Cooper & Morrison, 1978; Curd & Cooper, 1978).

1.7 FORMATION AND STRUCTURE OF THE CLASSICAL PATHWAY C3 CONVERTASE

C4 is a 200 kD glycoprotein consisting of three disulphide-linked polypeptide chains, α (90kD), β (80kD), and γ (30kD). The α chain possesses an internal thiolester (Tack, 1983) which, upon enzymatic removal of C4a (6 kD) from the N-terminus of the α chain (Schreiber & Muller-Eberhard, 1974) by $\overline{\text{C1}}$ or $\overline{\text{C1s}}$, becomes the metastable binding site of nascent C4b (Muller-Eberhard & Lepow, 1965). This binding site is unstable, and failure to bind to an antigen-antibody complex or other surface within milli-seconds results in loss of the binding site, and the molecule becomes inactive fluid-phase C4b. It has been suggested that only 1-2% of C4b molecules bind to the target, the remainder react with water in the fluid-phase to become inactive fluid-phase C4b. Binding of C4b involves the binding of the thiol group to a hydroxyl or amino group on the acceptor molecule to form either ester

TABLE 1.5

Non-Immunoglobulin activators of C1

Myelin basic protein

CRP complexes

Dextran sulphate

Lipid A

Heparin

Polyvinyl Sulphate

Nitrophenylated Molecules

Ant Venom Polysaccharide

Schistosoma Mansoni

Trypanosoma Brucei

E-Coli Bacterial Strains

Salmonella Bacterial Strains

or amide bonds respectively (Campbell et al, 1981).

C4b also carries two stable binding sites, one is the magnesium-dependent binding site for C2, and the other for the C3b/C4b receptor (CR1).

C4 occurs in the form of two structurally and functionally distinct isotypes, C4A and C4B (Carroll et al, 1984), which are coded by distinct genes which map to the class III region of the major histocompatibility complex, on the short arm of chromosome 6 (Carroll et al, 1984). C4A has a propensity to react with amino acids to form amide bonds, while C4B tends to bind to hydroxyl groups to form ester bonds (Law, Dodds & Porter, 1984).

C2 is a 110 kD glycoprotein, which is a single chain zymogen of the serine esterase family. On activation, C2 is cleaved into the N-terminal non-catalytic C2b (40 kD), and the catalytic C-terminal (70 kD) by C1s. Cleavage can occur in the presence or absence of C4b (Gigli & Austin, 1969). C2 binds to C4b via the C2b fragment, and remains bound to C2a by non-covalent bonds following proteolytic activation by C1s. (Nagasawa & Stroud, 1977).

The classical pathway C3 convertase is designated C4b2a. C3 convertase is an unstable enzyme having a very short half-life of 3 minutes at 30 C (Polley & Muller-Eberhard, 1968). C2a decays from the complex, which can be regenerated by fresh C2 in the presence of C1 or C1s.

C3

C3 is the precursor of many of the biologically active fragments that function by association with other complement proteins or by binding to cell-surface

receptors. It contains nearly ten distinct binding sites. It is a 190 kD glycoprotein, consisting of two disulphide-linked polypeptide chains, α chain (118 kD) and β chain (75 kD). Native C3 does not express binding sites for physiological ligands and thus does not exhibit biological activity. Proteolytic activation and processing are required to expose the sites which are concealed within the native molecule. Cleavage of the α chain by the classical or alternative pathway C3 convertases, releases a small peptide, C3a (9 kD) from the N-terminus. The remainder of the C3 molecule which is termed C3b, contains an internal thiolester bond. Following C3b formation, a conformational rearrangement occurs in the molecule which enables the thiolester to become reactive and form either ester bonds (by reactivity with hydroxyl groups) or amide bonds (by reacting with amino groups) on the acceptor surface. Thus C3b becomes covalently bound to the acceptor surface. If binding to the acceptor site does not occur within milliseconds, it reacts with water to become inactive fluid-phase C3b. It has been calculated that during complement activation only 5% of C3b binds covalently to the target, the remainder becoming the inactive fluid-phase product.

Both bound and fluid-phase C3b carry binding sites for B, H, P, C5 and CR1. The binding of C3b to the $\overline{C4b2a}$ complex, probably to C4b results in the formation of the classical pathway C5 convertase (Cooper & Muller-Eberhard, 1970), which is the first stage in the terminal sequence [Fig.1].

1.8 THE ALTERNATIVE PATHWAY

In the early fifties it was noted that many complex poly-saccharides such as zymosan or Gram-negative bacterial endotoxin were capable of consuming C3 without the utilization of C1, C4, or C2 (Pillemer et al, 1954). These observations were confirmed later by the demonstration that C3 consumption also occurred upon the addition of these polysaccharides to a C4 or C2 deficient sera (Frank et al, 1971; Johnson et al, 1972). They suggested the existence of an alternative pathway for complement activation, which was independent of C1 activation. In addition to polysaccharides, certain immunoglobulins and their F(ab')₂ fragments (Schur & Becker, 1963), guinea pig IgG1, and the human myeloma proteins IgG4, IgA1 and IgA2 (Sandberg et al, 1971; Sandberg & Osler, 1971; Osler & Sandberg, 1973) were shown to activate the alternative pathway of complement. Four proteins are involved in the assembly of the alternative pathway C3 and C5 convertases, namely C3, B, D, and P [Fig 1.1], under the control of two fluid-phase regulatory proteins, factors I and H.

The alternative pathway C3 convertase is formed when C3b binds to factor B in the presence of magnesium ions to form C3bB, which has weak C3 convertase activity (Medicus, Gotze & Muller-Eberhard, 1976). Factor D, a 23.5 kD serine protease, then cleaves B with the release of a 30 kD fragment Ba which results in the formation of C3bBb, the unstable fluid-phase alternative pathway C3 convertase. This convertase is unstable as Bb decays rapidly from the complex. At this stage the enzyme can be regenerated by the

addition of fresh B in the presence of D (Fearon et al, 1973). The enzyme $\overline{\text{C3bBb}}$ is stabilized by the binding of P to the complex, which results in the retardation of Bb decay (Fearon & Austen, 1975). $\overline{\text{C3bBbP}}$ is called the properdin-stabilised alternative pathway C3 convertase. The C3b-dependent positive feed-back loop of the alternative pathway is now established and each newly generated C3b molecule has the potential to form another convertase molecule by reacting with B and D [Fig 1.1]. If uncontrolled, this activation would continue until the supply of C3 or B was exhausted. The feedback loop is controlled by two proteins, factor I, a serine protease, and its cofactor, factor H. Acting in concert, these two proteins limit convertase formation by degrading C3b, and accelerating the decay of the convertase (see below). In the absence of either of these proteins, uncontrolled turnover of the alternative pathway occurs (Whaley & Thompson, 1977). With such efficient control proteins it is surprising that the alternative pathway is ever activated. However, it is now known that initiation of the alternative pathway occurs continuously by low-grade hydrolysis of the thiolester bond of C3. The product of this reaction ($\text{C3}^{\text{H}_2\text{O}}$) has properties like C3b, and is therefore called C3b-like-C3 (Pangburn, 1986). $\text{C3}^{\text{H}_2\text{O}}$ can bind factor B before being able to bind factor H. Thus a low efficiency fluid-phase C3 convertase is formed, which cleaves to form C3b which can bind to acceptor sites on surfaces in its immediate vicinity. If the C3b binds to a non-activating particle, factor H binds and the C3b becomes inactivated by factor I (see 1.12). However, if the C3b

binds to an alternative pathway activating surface, it becomes relatively resistant to factor H binding and so binding of factor B and the formation of the C3 convertase, C3bBbP, is favoured. Thus alternative pathway activators are really amplifiers of pre-existing low-grade fluid-phase turnover, rather than being true activators. As a consequence of alternative pathway activation, bacteria are opsonised by C3b and the formation of C5 convertase prepares them for lysis by the terminal membrane attack complex.

For C5 convertase formation to occur, a second molecule of C3b must bind to the C3 convertase, probably to the first C3b molecule which comprises part of the C3 convertase (Takata, et al., 1987). The alternative pathway C3 and C5 convertases cleave C3 and C5 at exactly the same sites as their classical pathway counterparts.

1.9 THE TERMINAL SEQUENCE

Cell lysis occurs as a result of the classical or alternative pathway activation via the formation of the membrane attack complex (MAC) (Bhakdi & Trandum-Jensen, 1983).

1.10 PROTEINS AND STRUCTURE OF THE MAC

C5 is a glycoprotein of 210 kD, consisting of two disulphide-linked polypeptide chains, α (130 kD) and β (80 kD). C5 is a thermo-labile molecule, as its activity is lost on heating at 56 °C for 10 minutes. The α chain is cleaved by the C5 convertase or trypsin with the release of 12 kD fragment, C5a, from the amino-terminus. C5a is an anaphylatoxin which also has chemotactic properties. The remainder of C5 is called C5b which carries a labile binding site for all cell membranes. Binding is promoted by the presence of C3b. The binding site is not covalent, which distinguishes it from the binding of C4b and C3b (Tack, 1980). Loss of the labile binding site follows a conformational change in the molecule to form a more stable fluid-phase structure. The functional activity of solid-phase C5b decays rapidly, unless it is stabilised by binding of C6.

C6 and C7 are very similar proteins, each consisting of a single polypeptide chain with molecular weight of 128 kD and 121 kD respectively. Both are β -globulins and contain some degree of α helical structure (Podack, Kolb & Muller-Eberhard, 1976). As mentioned above, C6 stabilises the membrane binding site of C5b, and fluid-phase C5b6

complexes can bind to cells which are distant from the site of complement activation. Such cells are susceptible to lysis by the subsequent binding of C7, C8 and C9, (Bystander lysis) (Lachmann & Thompson, 1970). The binding of C7 to membrane-bound C5b6 stabilises the binding of the complex to the membrane. This is due to the hydrophobicity of C7 which becomes inserted into the lipid bilayer (Podack, Klob & Muller-Eberhard, 1979).

C8 is a 150 kD β -globulin, which consists of three polypeptide chains, α (77 kD), β (63 kD), and γ (14 kD). The α and γ chains are held together by disulphide bonds, whereas the β chain is linked to them by non-covalent forces. The β subunit of which C8 binds to C5b in the absence of C6 or C7, while the C8 chain is intensely hydrophobic and penetrates the lipid bilayer. The formation of cell bound C5b-8 causes significant membrane damage and low-grade lysis may occur (Stolfi, 1968). C8 possesses a binding site for C9 (Bhakdi & Trandum-Jensen, 1982).

C9 is the terminal component of the complement system. It is a 79 kD β -globulin, which consists of a single polypeptide chain (Hadding & Muller-Eberhard, 1969). C9 has the tendency to polymerise, particularly when bound to C8. This property plays a central role in cell lysis (Tschopp, 1984).

MAC-polymerised C9 forms cylindrical structures which are heterogeneous in size, the inner diameters ranging from 9-12 nm (Tschopp, 1984). The MAC in its final form consists of a hydrophobic outer layer which becomes inserted deeply into the membrane lipids, while the

hydrophilic core provides a transmembrane channel, which allows the passage of ions and water (Mayer, 1972). The insertion of large numbers of MACs into cell membranes disrupts the membranes by distortion of the lipid bilayers leading to leaky-patches, and resulting in cell lysis (Esser et al, 1979).

1.11 REGULATION OF COMPLEMENT ACTIVATION

The complement system is under the control of two groups of regulatory proteins.

1.12. THE FLUID-PHASE REGULATORY PROTEINS

Levy and Lepow (1959), first described a heat-labile, non-dialysable substance in human serum which inhibited the hydrolytic activity of C1s on synthetic amino acid esters. C1-inhibitor (C1-inh) is an alpha-2 globulin with an approximate molecular weight of 110 kD. The protein is a single polypeptide chain, and is thermolabile (Harpel & Cooper, 1975).

C1-inh forms an equimolar complex with both activated $\overline{\text{C1s}}$ and $\overline{\text{C1r}}$, irreversibly blocking the activities of these proteases and preventing activation of C1s by $\overline{\text{C1r}}$ or cleavage of C4 and C2 by $\overline{\text{C1s}}$. The blocking occurs whether the subcomponents of C1 are free or incorporated in the C1 macromolecular complex. However, C1-inh does not form a stable complex with the precursor forms of these enzymes, or enzymes which have been inactivated with DFP (Ziccardi & Cooper, 1976). C1-inh also inhibits plasmin, kallikrein, activated plasma thromboplastin antecedent, and activated Hageman factor (Forbes, Pensky & Ratnoff, 1970; Ratnoff et al, 1982).

C4BP is a 10.0S glycoprotein with an apparent molecular weight of 590 kD, consisting of identical subunits of 70 kD molecular weight each, linked by disulphide bridges. The protein appears to be multivalent, with the capacity to bind as many as five or six C4b

molecules (Scharfstein et al, 1978).

In the presence of C4BP and factor I, C4b is cleaved at two points in the α chain yielding three peptides, while the β and γ chains remaining intact (Fujitani, Gigli & Nussensweig, 1978).

One of the α chain peptides is C4d, which is separated from the remainder of the molecule during the reaction. The other two peptides are covalently linked to the β chain and remain part of the C4c fragment. In the case of C4b bound to IC, C4c is released into the fluid-phase, indicating that the thioester by which C4b binds to IC is located within the C4d portion of the α chain.

Factor I is a serine esterase which is stable at 56 °C for four hours. The molecule consists of two disulphide-linked chains of 50-55 kD and 38-42 kD (Fearon, 1977). Factor I has no effect on native fluid-phase C3 or C4. However, in the presence of its cofactors, factor H or C4BP, it will enzymatically degrade C3b and C4b respectively. Although membrane-bound C3b and C4b are degraded by factor I in the absence of cofactor activity, the rate of reaction is much reduced (Whaley & Ruddy, 1976; Pangburn & Muller-Eberhard, 1983). In the presence of factor H, I cleaves the α chain of C3b in two closely adjacent sites. The resulting molecule, C3bi, has lost its ability to bind B and C5 but has acquired the ability to bind to specific C3bi receptors (CR3). C3bi consists of two α chain fragments (molecular weights of 68kD and 43kD) and a small fragment (3kD) is released. The two major α chain fragments remain bound to the intact β chain by disulphide bonds. In serum, C3bi is very slowly converted to a

fragment termed α 2D and C3c (Medof et al, 1982). α 2D (C3dg) consists of C3d and a second fragment, C3g, which can be released by trypsinisation (Lachmann, Pangburn & Oldroyd, 1982). The serum protease which converts C3bi to C3c and α 2D has not been identified, but plasmin remains a possibility (Chaplin, Monroe & Lachmann, 1982). There is evidence that factor I will convert C3bi to α 2D and C3c in the presence of its second cofactor CR1 (Medof et al, 1982). The observation that serum contains small quantities of CR1 (Yoon & Fearon, 1985) raises the possibility that factor I and serum CR1 are responsible for the final degradation of C3bi in serum.

α 2D (molecular weight 40kD) is the fragment of the chain which contains the thiolester of C3b. Thus α 2D remains covalently linked to C3b acceptors following C3b degradation. C3c (molecular weight 140kD) is released into the fluid-phase and has no known biological activity. This fragment consists of the intact β chain and two chain fragments (molecular weight 25kD and 43kD) respectively.

The most important consequences of inactivation of C3b by factor I is the loss of the ability of C3b to bind factor B or C5. Thus, the formation of the alternative pathway amplification C3 convertase, and the classical and alternative pathway C5 convertases, is inhibited.

Factor H is a heat-stable (56°C for 30 min) B1/ γ glycoprotein containing more than 15% carbohydrate, which consists of a single polypeptide chain of 150 kD (Whaley & Ruddy, 1976). Factor H is a cofactor which is essential for the full expression of the proteolytic activity of

factor I.

Factor H binds to C3b (Whaley & Ruddy, 1976). The binding affinity of factor H for C3b is approximately four times higher than that of factor B for C3b (Kazatchkine, Fearon & Austen, 1979). Thus, under normal conditions regulation of the alternative pathway occurs, as any factor B bound to C3b will be displaced by factor H. In addition to dissociating factor B from C3b, factor H acts as a cofactor for factor I, potentiating the conversion of C3b to C3bi which can no longer bind factor B.

The S protein is a 80 kD peptide chain, which is the primary MAC inhibitor in serum and functions by competing with the membrane binding site on C5b-7 to prevent membrane insertion. (Bhakdi & Tranum-Jensen, 1982). It has also been shown that the S-protein inhibits C9 polymerisation, (Dhalbak & Podack, 1985). On the other hand, C9 polymerisation per se has been found to be a mechanism for inactivating C9 (Dankert, Shriver & Esser, 1985). Recently it was confirmed that the S-protein is identical with the serum spreading factor, vitronectin (Jenne & Stanley, 1985).

1.13 THE MEMBRANE PHASE REGULATORY PROTEINS

Some of the membrane bound regulators have been relatively well characterised. Complement receptor 1 (CR1), decay-accelerating factor (DAF) and glycoprotein 45-70 (gp45-70) all bind C3b and C4b and thus are regulators of the early components.

CR1 is a single chain membrane glycoprotein with four identical allotypes (160 - 250 kD) which is found on erythrocytes, polymorphonuclear leukocytes, monocytes, macrophages, B-lymphocytes, a sub-population of T lymphocytes, dendritic cells and renal glomerular podocytes (Fearon 1979; Medof & Nussenzweig, 1984). CR1 binds C3b and serves as a co-factor for factor I in C3bi formation and possibly in the subsequent conversion of C3bi to C3c and Δ 2D. Furthermore, like factor H, CR1 displaces factor Bb from C3bBbP, but in addition CR1 also accelerates the decay of C4b2a, by displacing C2a (Seya, Holers & Atkinson, 1985). This regulatory effect is seen particularly when the convertases are in the fluid-phase, or on neighbouring cells. The effect is not so apparent if the convertases are bound to the cell membrane where CR1 is also bound.

It has been shown that CR1 and C5 compete for the same binding site on C3b (Fishelson, Schreiber & Muller-Eberhard, 1985) and it is therefore probable that CR1 also participates in the regulation of C5 convertase. In addition to these regulatory functions, CR1 serves as a cell membrane receptor for C3b-coated ligands (Ross & Medof, 1985).

DAF is an intrinsic human erythrocyte membrane

glycoprotein with a molecular weight of 70 kD (Nicholson-Weller et al, 1982). It is found on erythrocytes, platelets and all types of leucocytes. It has been found to accelerate the decay of the C3 convertases via binding to the catalytic subunits Bb and C2a. However, DAF also binds to C3b and C4b alone and may thereby prevent the formation of C3 convertases (Medof, Kinoshita & Nussenzweig, 1984; Fujita et al, 1988). In contrast to CR1, DAF acts mainly on the membrane where it is located. Thus CR1 and DAF act together to protect autologous cells against attack by complement.

Gp 45-70 binds C3b and C4b alone or in the convertase forms, and serves as a co-factor for factor I (Holers et al, 1985). DAF has no cofactor activity whereas CR1, DAF, and gp45-70 regulate complement activation on the cell membrane at the level of the C3 and C5 convertases.

The homologous restriction factor (HRF) (Schonermark et al, 1986; Zalaman, Wood & Muller-Eberhard, 1986) is also a membrane-bound regulator and serves to control the final assembly of the terminal complement complex and therefore act at a later stage to protect autologous cells against complement mediated lysis. HRF is a single chain plasma protein with molecular weight of 80 kD with an internal disulphide loop.

HRF, which has been demonstrated on erythrocytes and peripheral blood leucocytes, binds to C8 and C9, thereby inhibiting the transmembrane channel formation.

1.14 BIOLOGICAL ACTIVITIES OF THE COMPLEMENT SYSTEM

The complement system plays a key role in the inflammatory process, largely by virtue of the properties of many of its components or their activation products. The cytolytic consequences of complement activation first drew attention to the existence of the complement system. It is now known that activation of the complement system can cause lysis of nucleated and non-nucleated mammalian cells, bacteria, platelets, mycoplasma and viruses.

Cleavage of C4, C3 or C5 by their respective convertases or proteolytic enzymes, yields the anaphylatoxins C4a, C3a and C5a respectively. (Chenoweth, Cheung & Henderson, 1983), C5a being the most potent anaphylatoxin and C4a the least. Anaphylatoxins bind to receptors on the membranes of mast cells and basophils with resulting degranulation. These granules contain vasoactive amines such as histamine, and their release is associated with increased vascular permeability.

Smooth muscle contraction is induced by the direct action of anaphylatoxins on specific smooth muscle cell receptors and is independent of their histamine releasing activity (Regal, Eastman & Pickering, 1980). Anaphylatoxin inactivator (carboxypeptidase N) removes the N-terminal arginine from these molecules. The derivatives which are termed C4a des Arg, C3a des Arg, and C5a des Arg respectively, have no anaphylatoxic activity (Damerou et al, 1980).

C5a, but not C3a or C4a, is chemotactic for polymorphonuclear leucocytes or macrophages (Damerou,

Grunefeld & Vogt, 1978). The chemotactic activity is preserved following degradation of C5a by anaphylatoxin inactivator, although in this case C5a des Arg requires a serum co-factor to express its full chemotactic activity (Perez et al, 1980).

It is well known that complement reacted micro-organisms or IC are coated with complement activation products, mainly C3b. The presence of CR1 (complement receptor type 1) on phagocytic cells promotes phagocytosis and killing of micro-organisms (Leigh et al, 1979), and the elimination of IC. Other complement receptors are present on a variety of cells. CR2 (C3d receptor), CR3 (C3bi receptor), C1qR (C1q receptor), and the factor H receptor have all been identified, and structural and functional studies are well underway. Recent evidence supports the notion that CR1 and CR2 on lymphocytes may play a significant immunoregulatory role (Ross & Medof, 1985; Weiss et al, 1987; Cooper et al, 1988).

A peptide derived from the α -chain of C3 causes an initial leukopaenia followed by leukocytosis (McColl et al, 1974). This peptide, termed C3e has a molecular weight 10-12 kD and appears to be produced as a result of proteolytic cleavage of C3c by an unidentified plasma protease (Ghebrehiwet & Muller- Eberhard, 1979). In addition, C3e also stimulates the secretion of lysosomal enzymes from neutrophils (Ghebrehiwet, 1984).

C3d-k is another C3 fragment produced by the proteolysis of C3bi by kallikarein, which releases leukocytes from the bone marrow, and inhibits T-cell proliferative responses (Meuth et al, 1983).

1.15 SOLUBILIZATION OF IMMUNE PRECIPITATES

When antigen and antibody are mixed with buffer in a test tube, precipitation occurs. The amount of precipitate is dependent upon the proportions of antigen and antibody. Maximal precipitation occurs when the ratio of antigen to antibody is optimal, and this is defined as the equivalence point. At this point all the antibody has been precipitated. The amount of precipitate is reduced when the proportion of antigen to antibody is increased (antigen-excess). When the ratio of antigen to antibody is decreased (antibody-excess) precipitation of antigen still occurs. If immune precipitates are incubated at 37 °C with fresh serum, the amount of precipitate decreases. This apparent loss of precipitation is not due to enzymatic degradation of either antigen or antibody, but rather the complex becomes smaller and more soluble (Czop & Nussenzweig, 1975). This process, which has been termed solubilization, was shown to be complement-dependent from the observations that sera which had been decomplexed by heat-inactivation (56 °C for 30 min.), by EDTA treatment, or by incubation with zymosan or cobra venom factor, did not exhibit this property (Miller & Nussenzweig, 1975). An absolute requirement for an intact alternative pathway was demonstrated by the observation that sera which had been depleted of factors D, B or P were unable to support solubilization unless the missing component was replaced (Takahashi et al, 1978). The importance of the alternative pathway was re-emphasised when it was demonstrated that solubilization of immune complexes (IC) proceeded in the

presence of the purified components C3, B, D, P, H and I (Fujita, Takata and Tamura, 1981).

The observation that solubilization did not occur in the absence of either of the regulatory proteins H and I, indicated the essential requirement for both of these components. In the absence of either control proteins, inappropriate fluid-phase turnover of the alternative pathway occurred, resulting in rapid depletion of C3. Although solubilization displays an absolute requirement for an intact alternative pathway, there is good evidence that the classical pathway plays a non-essential role, serving to accelerate and increase the efficiency of the reaction. This conclusion is based upon the observations that although solubilization occurred in C2- and C4-deficient sera, there was a delay in the onset of the process and the rate of solubilization was decreased (Czop & Nussenzweig, 1975). The reduced rate of solubilization of complexes formed with the F(ab')₂ fragments compared with complexes formed with IgG antibody also indicates a role for the classical pathway (Czop & Nussenzweig, 1975).

As solubilization proceeds normally in C5-deficient or C6-deficient sera (Takahashi et al, 1976) but not in C3-deficient sera (Czop & Nussenzweig, 1975), it can be concluded that C3, but not the terminal components, are essential for the reaction. Solubilized IC can be precipitated with antiserum to C3, showing that they contain bound C3 (Takahashi, Takahashi & Hirose, 1980). Analysis of C3 has revealed that it is covalently bound to both the antibody and antigen moieties (Takata, Tamura, and Fujita (1984). Solubilization proceeds through three

distinct stages. Firstly, C3b is deposited on the surface of the IC. Under normal circumstances, when the antibody in the IC is capable of binding and activating C1, the initial deposition of C3b occurs very rapidly via the classical pathway (Takahashi et al, 1978). When the complexed antibody is incapable of activating C1, or if the serum is deficient in one or more of the classical pathway components, alternative pathway activation is responsible for the initial deposition of C3b. As this occurs more slowly, the solubilization process is prolonged (Takahashi et al, 1976).

Following the initial deposition of C3b on the IC the phase of amplification begins. The alternative pathway C3 convertase, C3bBbP, is assembled on the IC and further C3 cleavage ensures increased deposition of C3b on the IC. When insoluble IC are incubated with serum for a limited period and then washed they remain insoluble, but when purified C3 is added solubilization occurs expeditiously (Takahashi, Tack & Nussenzweig, 1971). Thus, the incorporation of C3b into the antigen-antibody lattice is the critical event in the solubilization process, and kinetic studies have clearly demonstrated that it precedes solubilization (Takahashi, Tack & Nussenzweig, 1977; Takahashi et al, 1978).

The C3b which is deposited by the classical pathway is clearly insufficient to produce this effect, but for reasons which are as yet unclear. Perhaps the amount of C3b deposited by the classical pathway is insufficient in quantity or is too limited in distribution to achieve

solubilization. The final phase of the solubilization process occurs after large quantities of C3b have been incorporated into the antigen-antibody lattice. This stage occurs in salt-free solution, in the absence of serum proteins and in the presence of chelating agents (Takahashi, Tack & Nussenzweig, 1971). As enzymatic degradation of neither antigen nor antibody occurs, it is thought that solubilization takes place because C3b disrupts the antigen-antibody lattice. Additionally, it is thought that the interference with Fc-Fc interactions, which play a major role in immune precipitation (Nisonoff & Pressman, 1958), is a critical event in solubilization as the Fab fragment of antibodies directed against the Fc piece of the complexed antibody can solubilize immune precipitates.

The observation that insoluble IC formed with the low affinity antibody were more easily solubilized than those formed with high affinity antibody suggests that disruption of antigen-antibody bonds may also play a role in solubilization (Czop & Nussenzweig, 1975). However, the evidence that antigen and antibody are dissociated is conflicting (Strassen & Beck, 1979). Of course, even if dissociation of antigen and antibody does not occur, it is possible that disruption of antigen-antibody bonds may play a role in the re-arrangement of the IC by preventing the reassociation and cross-linking of antigen. It is also conceivable that the binding of C3b may also modify the overall solubility of IC, in addition to the possible actions described above.

Because solubilization is a general phenomenon which

occurs under any physiological conditions, with any class of antibody and any soluble antigen (Czop & Nussenzweig, 1975), it probably represents an important protective mechanism whereby large IC which are either formed or deposited in the tissues are removed. This suggestion has been supported by Bartolotti & Peters (1978), who showed that de complementation of rabbits with experimentally-induced serum sickness delayed the removal of IC from the kidney.

1.16 PREVENTION OF IMMUNE PRECIPITATION (PIP)

It is unlikely that IC are normally formed in vivo in the total absence of complement, thus the effect of complement on the early stages of formation of insoluble IC may be of greater biological significance than is solubilization (Schifferli, Bartolotti & Peters, 1980). Schifferli showed that IC formed at equivalence, or in antibody-excess in normal serum remained soluble for up to two hours. After this time IC formed at equivalence tended to precipitate, in contrast to those formed in antibody excess, which remained soluble. Heat-inactivated serum or EDTA-treated serum did not prevent immune precipitation, observations which implicated complement as being responsible for the effect. The finding that sera depleted of factors B, D, or P were able to prevent immune precipitation showed that the alternative pathway was not required (Schifferli, Bartolotti & Peters, 1980; Schifferli, Woo & Peters, 1982). However, after an hour precipitation began to occur. IC formed in sera depleted

of calcium ions by EGTA treatment showed an early phase of precipitation which was followed by a phase of resolubilization (Schifferli, Woo & Peters, 1982). As only the alternative pathway was able to function under these conditions, it can be concluded that the alternative pathway is concerned with maintaining IC in solution, whereas the classical pathway is important in preventing precipitation during the early stages of IC formation. As excessive immune precipitation occurred in sera deficient in C1q, C4, C2 and C3 (Schifferli, Bartolotti & Peters, 1980; Schifferli, Woo & Peters, 1982; Naama et al, 1983), but not in sera which lacked C5 or C7 (Naama et al, 1983), the absolute necessity for the classical pathway components through C3 was established, and the lack of requirement for the terminal components beyond C3 was confirmed. Thus, as with solubilization, C3 activation and binding must be the critical step in the prevention of immune precipitation (PIP).

The precise role of the individual complement components and the relative roles of the classical and the alternative pathways in PIP have been defined by studying immune precipitation in the presence of purified complement components (Naama et al, 1984; Naama et al, 1985). The rate of precipitation of IC formed in C1 was reduced, although the extent of precipitation was not altered (Naama et al, 1984). Thus, C1 forms the first line of defence against immune precipitation. C1-inhibitor dissociates C1, leaving C1q bound to the complexes. When C1-inhibitor was mixed with C1, complexes precipitated rapidly (Naama et al, 1984). It has been suggested that low frequency of immune

complex disease in patients with C1-inhibitor deficiency (who lack C4) is due to the unopposed protective effect of C1 (Schifferli, Steiger & Schapira, 1985).

Although C4 and C2 on their own, or together, do not influence the rate of precipitation of IC, when they are mixed with C1 and C3 at the same concentrations present in serum, immune precipitation is prevented. The terminal C5-C9 components do not influence immune precipitation (Naama et al, 1984). In the presence of the six alternative pathway components (C3, B, D, P, H and I) an early phase of rapid immune precipitation occurred which was followed by resolubilization (Naama et al, 1984). Thus, the findings with complement-deficient sera and purified components demonstrated the absolute requirement for C3 in the PIP. The binding of C3b to IC is essential, and studies using 125

[I]-C3 have shown that the molar ratio of C3 to IgG antibody in the IC is consistently 2:5 (Naama et al, 1985). The molar ratio of C3b: IgG in solubilized IC is higher (1:1) (Fujita, Takata & Tamura, 1981) which indicates that greater amounts of C3b are required to solubilize immune precipitates than to prevent precipitation of IC. The site of attachment of C3b to immune complexes has not been ascertained, but presumably some must bind to the Fc region of the antibody to prevent Fc-Fc interactions. As mentioned earlier, during solubilization some C3b binds to the antigen and may disrupt some antigen-antibody bonds. Although the binding of C3 to antigen during PIP has not been demonstrated, there is no reason to believe that it does not occur. Such binding would help prevent extensive

lattice formation.

The outcome of the interaction of IC with complement is clearly influenced by properties of both antigen and antibody. Precipitation of IC formed with small protein antigens such as BSA was easily prevented, whereas IC formed with larger antigens, such as thyroglobulin, were relatively difficult to keep in solution (Whaley,1987). The affinity of antibody did not appear to affect the ability of complement to prevent immune precipitation (Johnson, et al.,1987). The nature of immunoglobulin isotype is critical in determining whether IC formed in serum will precipitate. Although IgG1 and IgM monoclonal anti-DNP antibodies produce IC which are rendered soluble extremely easily, IC formed with IgA precipitate rapidly even when formed in whole serum (Johnson, et al.,1987). Thus, properties of the antigens and antibodies presents in IC may play an important role in determining whether complexes become deposited within the tissues and produce immune complex disease.

Sucrose density gradient studies of IC which have been solubilized by the action of complement and IC which have been prevented from precipitation by the action of complement, have demonstrated that they exhibit heterogeneity of size, with sedimentation coefficients ranging from 11S to 22S (Czop & Nussenzweig, 1975; Malasit, Bartolotti & Humphrey, 1983).

In addition to C3, solubilized IC and IC prevented from precipitating by the action of complement contain C1q, C4, C4bp, C5 and factors B, P and H (Takahashi, Tack & Nussenzweig, 1977; Takahashi et al, 1978; Scharfstein et al, 1979; Campbell, Dodds & Porter, 1980; Naama et al, 1985).

Solubilized IC are unable to activate the classical or alternative pathways and have been termed 'end-stage' IC (Takahashi, Tack & Nussenzweig, 1977; Takahashi & Takahashi, 1981). However, complement-reacted IC can bind to staphylococcal protein A Sepharose (Takahashi & Takahashi, 1981). Solubilized IC can bind to erythrocyte CR1, but IC which have been formed in serum do not bind to this receptor terribly well (Schifferli & Peters, 1983). This difference can be explained on the basis that the number of CR1 receptors per erythrocyte is low (Fearon, 1980), and as multipoint attachment of C3b to receptors is required for stable binding to occur (Arnaout et al, 1981), large numbers of C3b molecules must be present on IC. As indicated earlier, greater amounts of C3b are bound to solubilized IC than to those which have been prevented from

precipitating. However, these results were obtained using BSA-anti-BSA IC and are not representative of all complexes. It is far more difficult to prevent the precipitation of IC formed with high molecular weight antigens such as thyroglobulin, although, when these IC are soluble they bind avidly to erythrocyte CR1 (Whaley, 1987). In this instance it is probable that more bound C3b is required to retain the IC in solution. The ability of complement reacted IC to bind to complement receptors is important. When the IC cannot be rendered sufficiently small and soluble by the action of complement, binding to erythrocyte CR1 must occur for the IC to be transported to the reticuloendothelial system (Cornacoff et al, 1983). The presence of C3b on IC also promotes their uptake and degradation by macrophages (Kjilstra, Van Es & Daha, 1979).

1.17 SOLUBILIZATION AND PREVENTION OF IMMUNE PRECIPITATION IN IMMUNE COMPLEX DISEASE

If the processing of IC by complement plays an important role in the protection of the individual against IC-mediated tissue injury, then breakdown of this mechanism should be evident in patients with immune complex disease. The most obvious example is the high incidence of immune complex disease in patients with inherited deficiencies of complement components. The most important observation is that immune complex diseases are principally associated with deficiencies of the classical pathway proteins, and patients who lack factors D or P do not appear to be unduly predisposed to the development of immune complex disease.

These experiments of nature illustrate the essential role of the classical complement pathway in defence against immune complex disease. By implication, PIP is more important than the solubilization of immune precipitates.

The majority of patients with immune complex diseases do not have inherited deficiencies of complement components, so other defects in the system must occur.

Acquired hypocomplementaemia occurs in patients with deficiencies of C1-inhibitor, and factors H or I. The former patients have uncontrolled catabolism of C4 and C2, with low levels of these components (Frank, Gelfand & Atkinson, 1976), and acquired C3 depletion occurs in patients with deficiency of H or I (Thompson, 1987). Patients with C1-inhibitor deficiency have a somewhat increased risk of developing SLE, but although C4 levels are usually extremely low the incidence of immune complex disease is far lower than that observed in inherited C4

deficiency. In the latter patients the presence of C1-inhibitor accelerates the aggregation of IC as a result of C1 disassembly. In contrast, in C1-inhibitor deficient patients the lower levels of C1-inhibitor may allow greater expression of the protective influence of C1 (Schifferli, Steiger & Shapira, 1985). Moreover, the presence of small amounts of C4 in the circulation of these patients might also help prevent the assembly of large IC aggregates.

Acquired hypocomplementaemia due to circulating C3 nephritic factor and the classical pathway convertase stabilizing factors are associated with pronounced hypocomplementaemia and immune complex disease (Daha, 1987). Hypocomplementaemia which occurs as a result of disease, may itself lead to further difficulty in handling IC. The prolonged low levels of C4 which may occur prior to clinical evidence of increased disease activity in SLE patients (Ruddy et al, 1971) and the hyposynthesis of C3 which occurs in SLE (Ruddy et al, 1975), may contribute to the difficulty in handling IC.

Recent investigations have shown that the sera of patients with a variety of immune complex diseases (Table 1.6) contain a factor which inhibits complement-mediated PIP (Naama, Mitchell & Whaley, 1983; Kerr, Naama & Whaley, 1986; Webb et al, 1986; Baatrup et al, 1983).

The presence of the inhibitory factor is associated with large PEG-precipitable IC. It is probable, therefore, that the factor which inhibits solubilization, and the factor which inhibits PIP are identical. The inhibitory factor appears to act by binding to IgG and preventing C1

TABLE 1.6

Immune-Complex Diseases Associated With Inhibition of PIP

- | | |
|----|------------------------------------|
| 1. | Rheumatoid arthritis |
| 2. | Systemic lupus erythromatosus |
| 3. | Sjogren's syndrome |
| 4. | Mixed connective tissue disease |
| 5. | Sub-acute infectious endocarditis. |

binding (Niven & Whaley, 1986), and activation (Naama, Mitchell & Whaley, 1983). Although purified monoclonal and polyclonal IgM-rheumatoid factors inhibit complement-mediated prevention of immune precipitation and solubilization (Whaley et al, 1987; Balastrieri et al, 1984; Mitchell et al, 1984; O'Sullivan, Amos & Williams, 1988), purified monoclonal IgM-myeloma proteins without rheumatoid factor are able to produce the same effects, although higher concentrations are required (Whaley et al, 1987). Furthermore, rheumatoid factor anti-idiotypic antibodies do not abrogate this inhibitory activity (Whaley et al, 1987), and serum concentrations of IgM rheumatoid factor did not correlate with levels of PIP inhibitory activity (Webb et al, 1986). Thus the precise role of IgM rheumatoid factor in the inhibition of PIP was questioned.

When all our IgM rheumatoid factor preparations were examined by sodium dodecyl sulphate polyacrylamide gel electrophoresis, it was found that all the preparations contained a contaminant with a molecular weight of 60kD. The possibility that this 60kD contaminant was responsible for inhibition of PIP was suggested. The purpose of this study was to isolate and characterise this 60kD "protein", and to assess its role in the inhibition of PIP.

CHAPTER 2

MATERIALS AND GENERAL METHODS

This chapter describes all the materials and general methods used.

2.1 MATERIALS

The following materials were obtained from the specified sources.

Pharmacia Ltd., Pharmacia House,
Midsummer Boulevard, Milton Keynes.

Sephadex G50

Sephadex G150

Sephacryl S-300 Super Fine High Resolution.

Cyanogen bromide (CN-Br) activated Sepharose 4B

Sepharose 4B

Protein A-Sepharose 4B

Con A-Sepharose 4B

Heparin-Sepharose 4B

Diethylaminoethyl (DEAE) Sephacel

Gel Bond

Sigma Chemical Co.Ltd., Poole, Dorset.

Bovine serum albumin (BSA)

Tween-20

N-Hydroxysuccinimido-Biotin.

Diaminobenzidine tetrahydrochloride

o-phenyldiamine

ϵ -amino-n-caproic acid

Trypsin, chymotrypsin, elastase, pepsin, protease V8, and

Sigma Chemical (continued)

neuraminidase.

Folin and Ciocalteu's phenol reagent

High and low molecular weight markers for SDS-PAGE.

Phenylmethanesulphonyl fluoride (PMSF)

monethanolamine

Sodium dodecyl sulphate (Lauryl sulphate)

Ammonium persulphate

N, N, N', N'-Tetramethylethylenediamine (TEMED)

Guanidine hydrochloride

Anisitol

Cyanogen Bromide.

Aldrich Chemical Co. Ltd., New Road,
Gillingham, Dorset.

Dimethyl sulphoxide

Benzamidine hydrochloride hydrate

Trifluoromethylsulphonic anhydride.

Amicon Ltd., Upper Mill, Stonehouse, Glos.

YM10 and YM30 filtration membranes.

Scottish Antibody Production Unit,
Law Hospital, Carluke, Lanarkshire.

Normal Rabbit serum

Sheep anti-whole human serum

Sheep anti-human IgG

Sheep anti-human IgM

Sheep anti-human IgA

Donkey anti-rabbit IgG

Peroxidase conjugated donkey anti-rabbit IgG

Sheep erythrocytes suspension

Regional Blood Transfusion Service, Law Hospital, Carlisle,
Lancashire.

Fresh frozen human plasma.

Atlantic Antibodies, ATAB, American Hospital supply Ltd.,
Didcot, Oxon.

IgG fraction of

Goat anti-human C1q

Goat anti-human C1r

Goat anti-human C1s

Goat anti-human C1-inhibitor

Goat anti-human C4

Goat anti-human C3

Goat anti-human C5

Goat anti-human factor B

Goat anti-human properdin

Goat anti-human factor H

Sheep anti-human serum albumin.

Cappel, Dynatech Laboratories Ltd.,
Deux Road, Billingshurst, Sussex.

Sheep anti-human C6

Sheep anti-human C7

Sheep anti-human C8

Behring Diagnostics, Hoechst UK Ltd., Hounslow,
Middlesex.

Sheep anti-human C4 binding protein

Sheep anti-human S-protein

Sheep anti-human prealbumin

Sheep anti-human transferrin.

Bio-Rad Laboratories Ltd., Coxton Way,
Watford, Hertfordshire.

Silver staining kit

Biorex-70 100-200 mesh (sodium free)

Difco Laboratories Ltd., P.O.Box 14B, Central
Avenue, East Molesley, Surrey.

Freund's complete adjuvant

Freund's incomplete adjuvant.

Amersham International PLC., Aylesbury,
Buckinghamshire, U.K.

C3a des Arg RIA kit

C4a des Arg RIA kit.

Sources for the following materials were:

Methanol: May and Baker Ltd., Liverpool Road, Manchester.

Diethylaminoethyl (DE52): Whatman Ltd., Springfield Mill,
Maidstone, Kent.

Ethanol: James Burrough, 60 Montford Place, London.

Anti-human C2: Seward Laboratory, Norse Road, Bedford.

Avidin-Peroxidase conjugate: Vector Laboratories Ltd.,
Bretton, Peterborough.

Kodak X.Omat XAR-5 films: Kodak, Hemel Hempstead, Herts.

BCA protein assay reagent: Pierce (UK) Ltd., Clifton Road,
Cambridge.

IgM anti-sheep erythrocytes: was a kind gift from

Dr R.A. Thompson,
Regional Immunology Dept.,
East Birmingham Hospital.

Human IgG1, IgG2, IgG3 and IgG4 subclass myeloma proteins:

were a kind gift from Dr.P.Bird, Immunology unit,
Pathology Department, Medical School,
Newcastle upon Tyne.

2.2 pH AND CONDUCTIVITY MEASUREMENT

pH : The pH of buffers and column fractions were measured using a Pye-Unicam pH meter, model 292.

CONDUCTIVITY: The conductivity of buffers was measured at 0°C using a conductivity meter model CDM3, Radiometer, Copenhagen.

2.3 BUFFERS

Phosphate buffered saline (20 x PBS.) pH 7.4

320 g NaCl

13.6 g Potassium dihydrogen phosphate

48.4 g di-Potassium hydrogen phosphate

Made up to 2 l with water.

Veronal buffered saline (5 x VBS) pH 7.4

85 g NaCl

3.75 g Barbitone sodium

5.75 g Barbituric acid

Dissolved in 1 l hot water, then made up to 2 l.

++
GVB pH 7.4

200 ml 5 x VBS

5 ml 0.03 M CaCl₂

10 ml 0.1 M MgCl₂

10 ml 10 % (w/v) gelatin

Made up to 1 l with water.

--
GVB pH 7.4

200 ml 5 x VBS

10 ml 10 % (w/v) gelatin

Made up to 1 l.

Isotonic dextrose (D5W⁺⁺) pH 5.5

50g D-glucose

5ml 0.03 M CaCl₂

10ml 0.1 M MgCl₂

Made up to 1l.

Isotonic 0.086 M EDTA pH 7.4

198ml of 0.3 NaOH was added to 500 ml of

0.12 M EDTA to give a pH of 7.4 . The volume
was then made up to 1 l with water.

0.04 M EDTA GVB⁻⁻

230 ml of isotonic 0.086 M EDTA was made up to
500 ml with GVB⁻⁻ .

0.01 M EDTA GVB⁻⁻

57.5 ml of isotonic 0.086 M EDTA was made up to
500 ml with GVB⁻⁻ .

D50S.

50g D-glucose

5.95g NaCl

Made up to 100 ml with water.

Mannitol GVB⁺⁺ .

9ml 20% Mannitol plus

3ml GVB⁺⁺

2.4 PROTEIN MEASUREMENT

a- O.D .
280

The absorbance of the samples were read at 280 nm using the sample buffer as a blank using a Shimadzu UV-120-02 spectrophotometer.

b- Folin assay : (Lowry et al.,1951)

known concentrations of BSA were used as a standards.

Solution A: 2g Na_2CO_3

20mg Na/K tartarate

made up to 100 ml with 0.1M NaOH.

Solution B: 0.15 % (w/v) $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$

Solution C: 1 volume of solution B was mixed with 50
volumes of solution A on the day of use.

Solution D: Commercial Folin and Ciocalteu's phenol

reagent was diluted with an equal volume of
water.

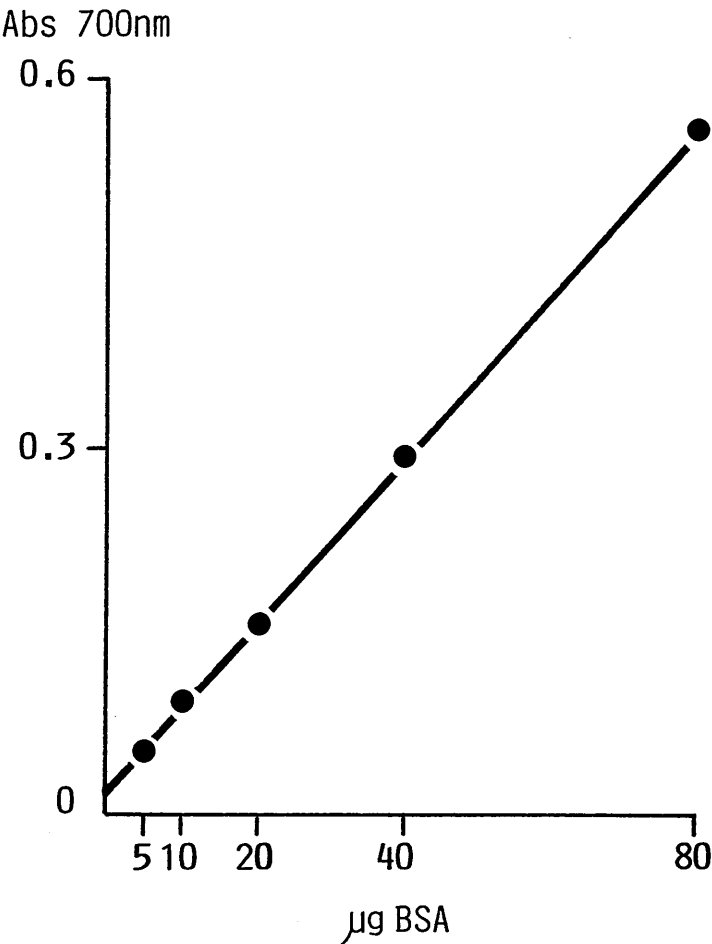
One hundred microlitres (100 μl) of standard dilutions of BSA (5 μg , 10 μg , 20 μg , 40 μg , 80 μg) or the test samples were each added to a series of test tubes containing 2 ml of solution C. After the addition of 200 μl solution D, the tubes contents were mixed and left to stand at room temperature for 30 minutes. The absorbance of each tube was read at 700 nm, and a standard curve (Fig 2.1) was constructed by plotting the O.D values of each standard dilution against the amount of BSA in that standard. The concentration of protein in the test samples were then read off from the standard curve.

Figure 2.1

Example of folin assay

standard curve

Folin assay standard curve



c BCA PROTEIN ASSAY REAGENT

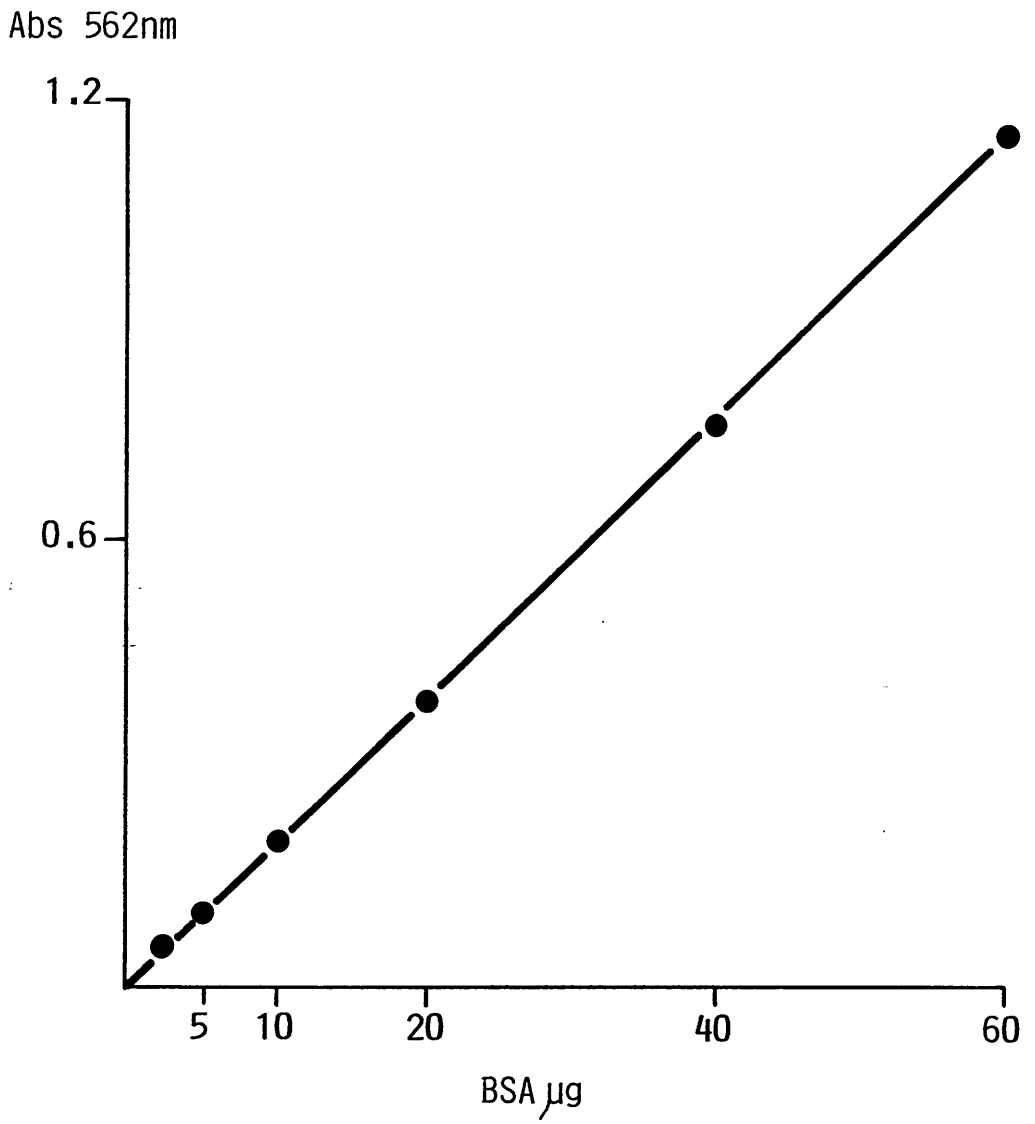
BCA working reagent: 1 volume of reagent B was mixed with 50 volumes of reagent A. One hundred microlitres (100 μ l) of standard dilutions of BSA or 100 μ l of test sample were each added to a series of test tubes containing 2ml of BCA working reagent. The tube contents were mixed and incubated at 60 °C for 30 minutes, after which the absorbance was read at 562 nm and a standard curve was constructed by plotting O.D. for each standard against the amount of protein contained in that standard (Fig 2.2). The amount of protein in test samples were then read off the standard curve.

Figure 2.2:

Example of BCA assay

standard curve

BCA standard curve



2.5 IMMUNOELECTROPHORESIS

(Grabar & Williams, 1953)

Tank buffer: 17 g Barbitone dissolved in 500 ml water
23.5 ml of 1 M HCl to give a pH of 8.6,
and the final volume was made to 11.

Agarose solution : 3 g agarose dissolved in 200 ml hot buffer
containing 0.9 g sodium barbitone, 2.3 ml
of 0.086 M EDTA pH 8.6.

10 x 10 cm Gel Bond membranes were cut, and 12.5 ml
of melted agarose was poured onto the hydrophilic side of
the membrane which had been placed on a level table. After
cooling at room temperature, a series of wells (2-4 mm) and
troughs were cut in the agarose using a template. The wells
were filled with the antigen or test sample together with
1ul Bromophenol blue (0.1 w/v) as a tracer, and the
electrophoresis was performed for 2-3 hours at a constant
current setting of 40 mA.

The electrophoresis was terminated when the dye had
migrated to the anodal end of the trough. After the troughs
were filled with the appropriate antiserum, diffusion of
antigen and antibody was allowed to occur for 24-48 hours
in a humid chamber at room temperature. The membranes were
then washed for 48-72 hours in 0.9% (w/v) NaCl, dried and
finally stained with 0.1% (w/v) Coomassie blue in 50%
methanol, 10% acetic acid, and destained in the same
solution without the dye.

2.6 RADIAL IMMUNODIFFUSION

(Mancini, Carbonara & Heremans, 1965)

Agarose (1g) was dissolved in 100 ml isotonic VBS (pH 7.4) containing 0.01M EDTA by boiling. When fully dissolved the agarose solution was cooled to 50 C in a water bath and the appropriate volume of antiserum added (the diameter of the precipitin ring for the 100% standard should be between 5 and 7 mm). After mixing, 3.5 ml was pipetted into each of plastic petri dish (50 mm diameter), which had been placed on a level surface.

The plates were then allowed to set at room temperature and stored inverted in a moist chamber at 2 C. For use, wells (2mm diameter) were cut in the agarose using a gel punch. Each well was filled with test sample and a series of three standards dilutions (1/1; 1/2; 1/4) were included in each plate. The plates were left for 24-48 hours in a moist box at room temperature after which the diameters of the precipitin rings were measured using a measuring magnifier.

A standard curve was constructed by plotting the square of the diameter of the precipitin ring for each standard against its protein concentration. The concentrations of the protein in the test samples were read from the standard curve. Test samples in which the protein concentration was too high to be read off the standard curve were diluted and re-tested.

2.7 DOUBLE IMMUNODIFFUSION (Ouchterlony, 1958)

Agarose (1% w/v) was prepared as described in section 2.6, but without the addition of antiserum. Gels were prepared on Gel Bond membranes as described in section 2.5. Wells (2-5mm diameter) were punched at variable distances from each other and adjacent wells were filled with antigen solutions and antisera. Diffusion was allowed to occur for 24 hours at room temperature in a moist chamber. The gels were then washed, stained and destained as described in section 2.5. (see specific sections in chapter 3 for details of each experiment).

2.8 SODIUM DODECYL SULPHATE-POLYACRYLAMIDE SLAB GEL ELECTROPHORESIS SDS-PAGE (Laemmli, 1970)

Protein samples were electrophoresed in different percentage gels made up according to Table 2.1

Stock solution	% Gel							
	6%	7%	8%	9%	10%	11%	12%	15%
30% Acrylamide (ml)	10	10	10	10	10	10	10	10
Water(ml)	15	11	8	6	5	3	2.5	2
0.75 M Tris-HCl pH 8.8 (ml)	25	21.5	19	17	15	14	12.5	10
Ammonium persulphate (mg)	50	42	38	33	30	27	25	24
10% SDS (ml)	0.5	0.43	0.38	0.33	0.3	0.27	0.25	0.24
TEMED (ul)	25	21	19	16	15	14	13	12

The stacking gel composition was as follows :

30% acrylamide	1.8 ml
Water	13 ml
0.75 M Tris-HCl pH 6.8	3 ml
10 % SDS	0.18 ml
Ammonium persulphate	18 mg
TEMED	9 μ l

The separation gel was poured immediately between glass plates (20 x 20 cm), separated by 1.0 mm spacers. Water was layered on top of the gel which was then left to set. After setting the water was poured off and the stacking gel was layered on top of the separating gel. A comb for forming wells at the top of the stacking gel was inserted and the gel was allowed to set for 2 hours at room temperature.

Tank buffer: 10ml 10% SDS
 33ml 0.75M Tris.
 14.2g glycine
 Made up to 1l with water.

Sample buffer: 3.00ml 10% SDS
 1.00ml glycerol
 0.85ml 0.75M Tris-HCl, pH 6.8
 0.50ml 2-mercaptoethanol
 100 μ l Bromophenol blue (0.01% w/v).

Samples containing 1 to 10 μ g protein were diluted with an equal volume of sample buffer (with or without mercaptoethanol; i.e under reducing or non-reducing conditions), and placed in a boiling water bath for 5 min.

The mixture was then loaded on to the gel and the electrophoresis performed at 40 mA for 2-3 hours until the tracking dye had reached the bottom of the gel. The gels were stained as in 2.9, 2.10, 2.11 or immunoblotted as in 2.12.

2.9 COOMASIE BRILLIANT BLUE STAINING

The slab gels were stained for 30 minutes in 500 ml of:

0.5g Coomassie Brilliant Blue

250ml Methanol

50ml glacial acetic acid

Made up to 500 ml with water.

Then the gels were destained in 10% methanol 5% acetic acid overnight, finally the gels were stored in water.

2.10 SILVER STAINING OF SLAB GELS

Using a silver staining kit, the gels were fixed for 30 min in 400ml of 40% (v/v) methanol, 10% (v/v) acetic acid, followed by another period in 400ml of 10% (v/v) ethanol, 5% (v/v) acetic acid. The gels were oxidised with 200 ml oxidizer solution diluted 1:5 with water for 5 min, followed by 3 washes with water (500 ml each).

Two hundred millilitre (200ml) of silver reagent solution (diluted 1:5 in water) was added, and the gel left on a shaker for 20 min. Following a short wash in 500 ml water, the developer (32 g/l) was added and finally the developing reaction was stopped by the addition of 400ml of 5% (v/v) acetic acid for 5 minutes.

Usually polyacrylamide gels were photographed wet, but some of those presented in this thesis were photographed after drying.

For drying the gel was placed on a cellophane membrane and dried in a Bio-Rad gel drier until absolutely dry.

2.11 GLYCOPROTEIN STAINING (PAS STAIN, CLARKE, 1964)

The gel was placed in 1l of 7.5% (v/v) glacial acetic acid for one hour at room temperature, followed by incubation in 500 ml 0.2% (w/v) periodic acid at 4 °C for a further hour. The gel was stained in 200ml Schiff's reagent for 1 hour at 4 °C, and finally destained in a solution containing 50 ml 1N HCl and 5 g potassium metabisulphite made up to 1l with water. The gels were stored in 3% (v/v) acetic acid at 4 °C.

2.12 PROTEIN TRANSFER BLOTTING (IMMUNOBLOTTING-WESTERN BLOTTING) (Towbin, Staehlin & Gordon, 1979)

Proteins were transferred from polyacrylamide slab gels to a nitrocellulose membrane at constant voltage of 16 V for 18 hours in a Bio-Rad Trans-Blot apparatus. The buffer used in the electroblotting cell was 24 mM Tris, 192 mM glycine (pH) containing 20 % (v/v) methanol.

2.13 IMMUNOSTAINING OF WESTERN BLOTS

The nitrocellulose membranes were incubated for 2 hours at room temperature in blocking buffer (10 mM Tris-HCl, 0.15 M NaCl, pH 7.5, containing 2 % (w/v) BSA, and 0.05% (v/v) Tween-20. The membranes were then incubated for two hours at room temperature with the IgG fraction of the

appropriate polyclonal antiserum at a concentration of between 10 ug/ml and 100 ug/ml in blocking buffer. Following six 10 minute washes in 250 ml blocking buffer, the membranes were incubated with peroxidase-conjugated donkey anti-rabbit IgG in blocking buffer for another 2 hours at room temperature. The membranes were then washed 6 times as described above, before developing with a solution of 50 mg diaminobenzidine tetrahydrochloride in 100 ml Tris-HCl (0.1M pH 7.6), containing 20 ul hydrogen peroxide (30 % v/v). The reaction was terminated by washing in water, and drying the membranes.

2.14 RADIOIODINATION OF PROTEINS

The following two methods were used for labelling proteins with ¹²⁵-[I] for the work described in this thesis.

2.14a Chloramine T method (McConahey & Dixon, 1966)

One millilitre (1ml) of protein solution (1 mg/ml) in PBS was mixed with 10ul of chloramine-T solution (5 mg/ml) and 38 MBq of carrier-free ¹²⁵-[I] for 60 seconds at room temperature. The reaction was stopped by the addition of 50 ul L-tyrosine (0.5 mg/ml in 0.1M NaOH) before the reactants were applied to a Sephadex G-50 column (0.5 x 8 cm) equilibrated in PBS, and a 0.5 ml fractions were collected. An aliquot (5 μ l) of each fraction was counted in an automatic-gamma counter (Packard, Model 300C) to determine the radioactivity.

The peak of radiolabelled protein was pooled, and the acid-precipitable radioactivity was determined by adding 10

μ l of the radiolabelled protein pool to 200 μ l of BSA (10% w/v), after which 1 ml of TCA (20% w/v) was added.

The contents of the tubes were then mixed and centrifuged at 10 000 g for 5 minutes. The supernatants were removed and the pellets were washed three times in 10% TCA prior to counting. In addition 5 μ l of the labelled preparation was counted to measure the total radioactivity in the preparation.

The percentage acid-precipitable radioactivity was calculated from the equation:

$$\% \text{ Acid-precipitable } = \frac{\text{total cpm} - \text{precipitated cpm}}{\text{total cpm}} \times 100$$

125-[I]-protein

The protein concentration of the radiolabelled preparations was determined by measuring the absorbance at 280 nm (2.4).

2.14b Iodogen method (Markwell & Fox. 1978)

A solution of 1,3,4,6-tetrachloro-3~~4~~,6~~5~~-diphenylglycoluril (Iodogen; 0.25mg/ml) in methylene chloride was prepared, and 50 μ l was placed in a glass tube and evaporated under a stream of nitrogen in order to form a thin layer of Iodogen on the wall of the tube. The protein (250 μ g/ml) in PBS, and 38 MBq carrier free ¹²⁵[I] were added, and the reaction was allowed to proceed at room temperature for 5 minutes with gentle mixing. The reaction was terminated by applying the reaction mixture to a Sephadex G-50 column (0.5x8cm) equilibrated in PBS or PBS containing 0.1% (w/v) gelatin. The protein was eluted with

PBS and 0.5 ml fractions were collected. The fractions were counted as in 2.14a and the peak of radiolabelled protein was pooled.

Acid-precipitable radioactivity was measured as described above. The protein concentration of proteins labelled by this method was determined by O.D ₂₈₀ (C1q, IgM-RF, IgG-RF) or by ELISA (PIP-inhibitor). Radiolabelled proteins were stored at -20°C in aliquots.

2.15 STANDARDIZATION OF ERYTHROCYTE SUSPENSIONS

A sheep erythrocyte suspension was washed three times in GVB until the supernatant was free of haemoglobin. One hundred microlitres (100 µl) were lysed in 2.9 ml of water, and the O.D at 541 nm was determined. The suspension was standardized using the following formula:

$$\text{Volume of E required} = \text{Initial volume} \times \frac{\text{O.D}}{0.385^*}$$

* O.D 0.385 at 541 nm equals 1×10^9 cells/ml.

These cells were used for the preparation of antibody coated erythrocytes, EA (see chapter 5, section 5.1.18), for the preparation of EAC₄ or EAC₁₄₂ haemolytic intermediates, the cell suspension was adjusted to 1×10^8 /ml, so in this case the optical density of the 1:30 lysate was read at 414 nm. A reading of 0.327 corresponds to a cell concentration of 1×10^8 /ml.

2.16 SEPARATION OF HUMAN OR RABBIT SERUM

Blood was collected into sterile universal glass
containers, and incubated at 37 °C for 30 minutes. to allow
clotting to occur, followed by 30 minutes on ice to allow
clot retraction to occur. The blood was centrifuged at 2000
g at 2 °C for 10 minutes, and the serum was collected and
divided into aliquots. Human serum was stored at -70
°C, while the rabbit serum was heat-inactivated at 56 °C
for 30 minutes prior to storage at - 20 °C.

CHAPTER 3

PURIFICATION AND PARTIAL CHARACTERISATION OF THE
INHIBITOR OF PREVENTION OF IMMUNE PRECIPITATION
(PIP-INHIBITOR) AND ANTISERUM PRODUCTION

INTRODUCTION

Previous attempts in this laboratory to purify the PIP-inhibitor from rheumatoid arthritis sera, usually resulted in preparations which contained mainly IgM.rheumatoid factor--(Mitchell et al.,1984). Since normal human serum contained a small amount of PIP inhibitory activity, it was hypothesised that the PIP-inhibitor was a plasma protein which was present in normal serum but which was present in much higher concentrations in rheumatoid arthritis sera. It was therefore decided to use normal human serum as starting material for its purification. The purification methods were originally based on the observation made by Niven and Whaley (1986), that the PIP-inhibitor binds to the IgG moiety of the IC, and involved affinity chromatography on IgG-Sepharose, protein A-Sepharose and Con.A-Sepharose or heparin-Sepharose. Later, a method which avoided IgG-Sepharose affinity chromatography was developed. The purified PIP-inhibitor was used to immunise rabbits to produce a monospecific antiserum.

METHODS

3.1.1 PURIFICATION OF IgG FROM WHOLE SERUM (Whaley et al, 1983)

The IgG fraction of normal animal or human serum was obtained by the addition of equal volume of saturated ammonium sulphate drop-wise with stirring at 0 ° C. The precipitate was collected by centrifugation (5000 g, for 30 mins. at 2 ° C), dissolved in 5 X VBS and dialysed for 18 hours against 10 mM Tris-HCl (pH 8.0.). The dialysed material was clarified by centrifugation (5000 g, for 30 mins. at 2 ° C) and applied to a DE52 column equilibrated in the same buffer. The column was then washed with buffer and the exclusion peak of the column was pooled. Following dialysis against PBS the preparation was stored at -20 ° C. The purity of the IgG was examined by SDS- PAGE run under reducing conditions in 10% gels and double diffusion in agarose gel against anti-whole serum and anti-IgG.

3.1.2 CONJUGATION OF IgG TO SEPHAROSE 4B (Goetzl & Metzger, 1970)

Five hundred millilitres (500 ml) Sepharose 4B were washed 3 times with an equal volume of deionised water, followed each time by centrifugation (2000g for 5 mins. at 4 ° C). The washed Sepharose was kept on ice and added to a solution of Cyanogen bromide (500 g/5l) in water with gentle stirring, and the pH was maintained around 11 by the addition of 4N NaOH, until the pH was stable at 11. The activated Sepharose was transferred to an ice-filled scintered-glass funnel attached to a vacuum-flask, and

washed with 50l of ice-cold 0.1 M sodium carbonate buffer (pH 8.2). The Sepharose was divided into 25 ml portions and to each was added an equal volume of IgG in the same buffer (ratio 10 mg protein: 1 ml Sepharose), and mixed end-over-end at 4 °C for 18 hrs. The supernatant was removed after centrifugation (200 g for 5 mins. at 2 °C), and following the measurement of the protein concentration (OD₂₈₀) the conjugation efficiency was determined using the formula:

% protein bound =

$$\frac{\text{O.D.* of starting material} - \text{O.D of supernatant}}{\text{O.D Of starting material}} \times 100$$

* O.D 280 nm

The remaining active sites on the Sepharose were blocked by incubation with 1 M mono-ethanolamine (pH 8.0) for 2 hours at room temperature, followed by three washing cycles of alternating pH. Each cycle consisted of a wash with glycine-HCl buffer (pH 3.0), containing 0.5 M NaCl, followed by a wash with 0.1 M Tris-HCl (pH 8.0) containig 0.5 M NaCl. The IgG-Sepharose was finally stored after the addition of sodium azide to a final concentration of 0.02% (w/v).

The same method was used for the preparation of BSA-Sepharose but it was scaled down to accommmodate one tenth of the volumes Sepharose and BSA (50 ml each).

3.1.3 RADIOLABELLING OF PROTEINS

Both BSA and the PIP-inhibitor were radioiodinated using the chloramine-T method for BSA and the solid-phase Iodogen method for the PIP-inhibitor (2.14a; 2.14b).

3.1.4 EQUIVALENCE POINT FOR BSA AND ANTI-BSA

The point of equivalence of ^{125}I -BSA and anti-BSA was determined by use of a quantitative precipitin test, where tubes were set up containing a constant amount of anti-BSA in 10 μl with decreasing concentrations of ^{125}I -BSA (600 $\mu\text{g}/\text{ml}$ to 9.4 $\mu\text{g}/\text{ml}$) in 5 μl GVB⁺⁺. The total reaction volume was adjusted to 115 μl with GVB⁺⁺.

After one hour incubation at 37^o C, 50 μl aliquot was removed and added to 1 ml ice-cold PBS, which was then centrifuged at 8000 g for 10 minutes and 250 μl of supernatant was counted in a gamma counter. Control tubes were made of ^{125}I -BSA and GVB⁺⁺ and processed in identical fashion to the test tubes. Percent immunoprecipitation was calculated using the following formula:-

% precipitation =

$$1 - \left(\frac{\text{c.p.m. buffer control} - \text{c.p.m. test}}{\text{c.p.m. buffer control}} \right) \times 100$$

Percentage precipitation was plotted against the concentrations of BSA and the equivalence point and the amounts of BSA at, four-times antigen and four-times antibody excess were determined. The anti-rabbit BSA antiserum used for the formation of the IC was kindly prepared by Mrs. A. Johnson. This antiserum was also used to prepare immunoadsorbent purified IgG anti-BSA (see 4.1.8).

3.1.5 ASSAY FOR INHIBITION OF PIP

Normal human serum (2.16) had previously been titrated in the PIP assay in order to determine the dilution which would inhibit precipitation of BSA-anti-BSA IC by between 70-90% (See 5.). For measurement of inhibition of PIP 50 μ l of serum diluted 1:4 in GVB⁺⁺ was mixed with 50 μ l of test sample or heat-inactivated serum, and ¹²⁵[I]-BSA (0.5 μ g in 5 μ l) was added. The reaction mixture warmed to 37 °C and at time 0, 10 μ l anti-BSA (diluted in GVB⁺⁺ in order to form IC at equivalence) was added and the incubation was continued at 37 °C. At timed intervals or after one hour, an aliquot (50 μ l) was removed and transferred to an Eppendorf tube containing 1ml ice-cold PBS. Following centrifugation for 5 min at 8000 g in a Beckman Microfuge B, 250 μ l of the supernatant was removed and the radioactivity counted in an automatic gamma counter. Control tubes included:

- i) radioactivity precipitated by antibody alone:-
100 μ l GVB⁺⁺, 5 μ l ¹²⁵[I]-BSA, 10 μ l anti-BSA.
- ii) Maximum PIP by serum alone:-
50 μ l GVB⁺⁺, 50 μ l serum, 5 μ l ¹²⁵[I]-BSA, 10 μ l anti-BSA.
- iii) Total radioactivity input:-
5 μ l ¹²⁵[I]-BSA, 50 μ l heat-inactivated serum, 60 μ l GVB⁺⁺

The control tubes were processed in an identical fashion to the assay tubes. Results were calculated using the formula:

$$\% \text{ Inhibition of PIP} = 1 - \left(\frac{\text{c.p.m test} - \text{c.p.m buffer}}{\text{c.p.m serum} - \text{c.p.m buffer}} \right) \times 100.$$

To investigate the kinetics of the action of PIP-inhibitor on the inhibition of PIP, the same assay was used but the PIP-inhibitor was added before the formation of IC in serum, or at timed intervals after their formation.

3.1.6 ASSAY FOR INHIBITION OF SOLUBILIZATION

BSA-anti-BSA immune precipitates were formed at equivalence by incubating $0.125 \mu\text{g}$ ¹²⁵[I]-BSA and $10 \mu\text{l}$ anti-BSA (1:8) in $25 \mu\text{l}$ GVB ⁺⁺ for 30 min at 37°C . $50 \mu\text{l}$ of test sample (dialysed in VBS), and $50 \mu\text{l}$ normal serum were added, and the incubation allowed to proceed for timed intervals or another hour. The soluble IC were separated as described in 3.1.5.

Controls included:-

- i) zero solubilisation:- $50 \mu\text{l}$ EDTA-serum in place of normal serum.
- ii) Maximum possible solubilization:- $50 \mu\text{l}$ GVB ⁺⁺ in place of test sample.

The results were expressed as % inhibition from the formula:

% Inhibition of solubilisation =

$$\left(\frac{\text{c.p.m Max. solubilisation} - \text{c.p.m test}}{\text{c.p.m Max. solubilisation} - \text{c.p.m zero control}} \right) \times 100$$

3.1.7 COLUMN SCREENING PROCEDURE

The column fractions were screened for inhibition of PIP (3.1.5), protein concentration (2.4), pH and conductivity (2.2).

3.1.8 PURIFICATION OF THE PIP INHIBITOR (METHOD 1)

The method consisted of affinity chromatography of normal serum on IgG-Sepharose, protein A-Sepharose and Con.A-Sepharose affinity chromatography.

All procedures, unless otherwise stated, were performed between 2^o to 4^o C.

3.1.9 IgG-SEPHAROSE AFFINITY CHROMATOGRAPHY

One unit of fresh frozen plasma was thawed at 37^o C, recalcified by the addition of 4 ml 1 M CaCl₂, cooled to 4^o C and allowed to clot for 18 hrs. After separation of the fibrin clot, the serum was diluted with an equal volume of 20 mM EDTA (pH 7.4), and 100 ml was applied to an IgG-Sepharose column (2.6 x 60 cm) equilibrated in 10 mM Tris-HCl buffer (pH 7.4), containing 10 mM EDTA and 75 mM NaCl, at a flow rate of 50 ml/hr. Fractions of 10 ml were collected.

The column was washed with the starting buffer until the O.D₂₈₀ of the effluent was less than 0.01 and then eluted sequentially with a column volume of each of the following buffers:-

10 mM Tris-HCl(pH 7.4) containing 10 mM EDTA and 0.15 M NaCl.

10 mM Tris-HCl(pH 7.4) containing 10 mM EDTA and 0.5 M NaCl.

2 M guanidine-hydrochloride.

The collected fractions were then dialysed against VBS for 18 hours, and screened as described in 3.1.7.

Fractions which inhibited PIP were pooled and subjected to protein A-Sepharose affinity chromatography.

3.1.10 PROTEIN A-SEPHAROSE AFFINITY CHROMATOGRAPHY

The PIP inhibitor pool (3.1.9) was applied to a protein A-Sepharose column (1 x 20 cm) which had been equilibrated in VBS. The column was washed with VBS at a flow rate of 30 ml/hour until the O.D₂₈₀ of the effluent was less than 0.01. The bound proteins were then eluted with 0.1 M glycine-HCl buffer (pH 2.8) containing 0.5 M NaCl. The acid-eluted fractions were neutralised by the addition of 100 ul of 1 M Tris base and dialysed overnight against VBS. The column fractions were screened as described in 3.1.7, and the exclusion peak which contained most of the PIP inhibitory activity was pooled and subjected to Con.A-Sepharose affinity chromatography.

3.1.11 CON.A-SEPHAROSE AFFINITY CHROMATOGRAPHY

The protein A-Sepharose pool was applied to Con.A-Sepharose (1 x 20 cm), equilibrated in VBS. After washing with VBS, the column was eluted with 0.1 M glycine-HCl buffer (pH 2.8) containing 0.5 M NaCl. The acid eluted fractions were neutralised and processed as described in 3.1.10. The PIP inhibitory fractions were pooled, concentrated by ultrafiltration using an amicon YM10 membrane and finally stored at - 70 °C.

The purity of the PIP-inhibitor was confirmed by SDS-PAGE run under reducing and non-reducing conditions in 10% gels (2.8), with silver staining (2.10), and by double diffusion in agarose gels (2.7) against anti-whole human serum.

3.1.12 PURIFICATION OF THE PIP-INHIBITOR (METHOD 2)

The method consisted of affinity chromatography of normal human serum on IgG-Sepharose, protein A-Sepharose, gel filtration and finally heparin-Sepharose affinity chromatography. At all stages of the preparation pools of PIP inhibitory materials were treated with 1 mM PMSF.

3.1.13 IgG-SEPHAROSE AFFINITY CHROMATOGRAPHY

Three units of fresh frozen plasma were clotted as described in 3.1.9. Benzamidine (10 mM), PMSF (1mM) and sodium azide (0.01% w/v) were added to the serum, and 500 ml was applied to IgG-Sepharose column (5.2x60 cm) equilibrated in 10 mM Tris-HCl buffer (pH 7.4), containing 10 mM EDTA, 0.15 M NaCl, 10 mM benzamidine, 1 mM PMSF and 0.01% (w/v) sodium azide, at a flow rate of 50 ml/hr. Fractions of 10 ml were collected. The column was washed with one column volume of the starting buffer, followed by several column volumes of 10 mM Tris-HCl buffer (pH 7.4), containing 10 mM EDTA, 1.0 M NaCl, 10 mM bezamidine, 1 mM PMSF, and 0.01% (w/v) sodium azide, until the O.D₂₈₀ of the column effluent was less than 0.01. Finally, the column was eluted with a column volume of 2 M guanidine-hydrochloride. The fractions were dialysed against VBS at 4 °C for 18 hours, and screened as described in 3.1.9. The fractions which inhibited PIP were pooled and subjected to protein A-Sepharose chromatography.

3.1.14 PROTEIN A-SEPHAROSE CHROMATOGRAPHY

The same method described in 3.1.10 was used although the Protein A-Sepharose column was larger (1 x 40 cm). The fractions were screened as described in 3.1.7, and those which inhibited PIP were pooled and concentrated by ultrafiltration at 0 °C using an Amicon YM10 membrane.

3.1.15 SEPHACRYL S300 HIGH RESOLUTION GEL FILTRATION CHROMATOGRAPHY

Five millilitres (5 ml) of the concentrated PIP inhibitor pool (3.1.14) were applied to 2.6 x 90 cm column of Sephacryl-S300 equilibrated in 10 mM Tris-HCl buffer (pH 7.4), containing 10 mM EDTA, 1 M NaCl, 5 mM benzamidine, 1 mM PMSF and 0.01% (w/v) sodium azide, at a flow rate of 15 ml/hr. Fractions of 2 ml were collected. The fractions were dialysed against VBS and were screened as described in 3.1.7. The fractions which inhibited PIP were pooled and dialysed against 50 mM Tris-HCl buffer (pH 7.5), containing 2 mM CaCl₂, and 2 mM benzamidine. The procedure was repeated until the entire concentrated PIP-inhibitor pool from the protein-A Sepharose column had been processed.

3.1.16 HEPARIN-SEPHAROSE AFFINITY CHROMATOGRAPHY

The dialysed PIP-inhibitor pool (3.1.15) was centrifuged (2000 g for 10 min at 4 °C), to remove insoluble material prior to being applied to a heparin-Sepharose column (1.5 x 20 cm) equilibrated in the dialysis buffer, at a flow rate of 25 ml/hr. Fractions of 5 ml were collected. After washing the column with starting

buffer, the bound proteins were eluted with a linear salt gradient consisting of 300 ml starting buffer and 300 ml starting buffer containing 1 M NaCl. The fractions were screened as described (3.1.7) and the purity of the PIP-inhibitor was confirmed as described in 3.1.11. The PIP inhibitor fractions were pooled and concentrated by ultrafiltration using an Amicon YM10 membrane and stored in aliquots at -70 ° C.

3.1.17 PURIFICATION OF THE PIP-INHIBITOR (METHOD 3)

The third method of PIP-inhibitor purification involved ion-exchange chromatography on DEAE-Sephacel, Biorex-70, gel filtration on Sephacryl-S300 and heparin-Sepharose affinity chromatography.

3.1.18 DEAE-SEPHACEL ION-EXCHANGE CHROMATOGRAPHY

One litre (1 l) of normal human serum was prepared as described in 3.1.10 and diluted with 10 mM sodium phosphate (pH 8.0), containing 10 mM benzamidine, 5 mM EDTA, 25 mM EACA, 1 mM PMSF and 0.01% (w/v) sodium azide, until the conductivity was 4 mS at 0 ° C. The pH was then adjusted to 8.0. The diluted serum (8.0 l) was applied to a DEAE-Sephacel column (5.6 x 90 cm) equilibrated in 10 mM sodium phosphate buffer (pH 8.0), containing 20 mM NaCl, 10 mM benzamidine, 5 mM EDTA, 25 mM EACA, 1 mM PMSF and 0.01% (w/v) sodium azide, at a flow rate of 300 ml/hr. Fractions of 20 ml were collected. Once the starting material had been washed through with starting buffer, the column was eluted with a linear salt gradient consisting of 5 l

starting buffer and 5 l of starting buffer containing 0.5 M NaCl. The column fractions were screened as described (3.1.7). Fractions which inhibited PIP were pooled, dialysed and applied to a Biorex-70 column.

3.1.19 BIOREX-70 CATION-EXCHANGE CHROMATOGRAPHY

The DEAE-Sephacel pool (3.1.18) was dialysed against several changes of 10 mM sodium phosphate buffer (pH 7.0) containing 20 mM NaCl, 5 mM benzamidine, 5 mM EDTA, 25 mM EACA, 1 mM PMSF and 0.01% (w/v) sodium azide, and applied to a Biorex-70 column (2.6 x 90 cm) equilibrated in the same buffer. A flow rate of 25 ml/hr was used and 10 ml fractions were collected. Once the exclusion peak had been washed through the column, the bound proteins were eluted with a linear salt gradient consisting of 2l of starting buffer and 2l of starting buffer containing 0.7 M NaCl. The fractions were screened as described (3.1.7), and those which inhibited PIP were pooled, concentrated by ultrafiltration using an Amicon YM10 membrane and applied to a Sephacryl-S300 column.

3.1.20 SEPHACRYL-S300 GEL FILTRATION CHROMATOGRAPHY

The same experimental details described in 3.1.15 were followed. The PIP inhibitory fractions were pooled, dialysed against 50 mM Tris-HCl buffer (pH 7.5), containing 2 mM CaCl₂ and 2 mM benzamidine, and centrifuged (2000 g for 1 minutes at 4 °C). The supernatant was subjected to heparin-Sepharose affinity chromatography.

3.1.21 HEPARIN-SEPHAROSE AFFINITY CHROMATOGRAPHY

The dialysed and centrifuged pool (3.1.20) was applied to a heparin-Sepharose column (2 x 15 cm) equilibrated in 50 mM Tris-HCl (pH 7.5), containing 2mM CaCl₂ and 2 mM benzamidine at a flow rate of 25 ml/hr. After washing the starting material in the column until the OD₂₈₀ of the effluent was less than 0.01, the bound proteins were eluted with 50 mM Tris-HCl buffer (pH 7.5) containing 10 mM EDTA, 1M NaCl and 2mM benzamidine. The fractions were screened and the purity of the PIP-inhibitor was confirmed as described in 3.1.12.

3.1.22 PRODUCTION OF ANTISERUM TO THE PIP INHIBITOR

Thirty micrograms (30 ug) of the purified PIP-inhibitor (prepared by method 1) were emulsified in 0.5 ml of Freund's complete adjuvant, and injected bilaterally into the popliteal lymph nodes of rabbits. Booster injections of 10 ug of the inhibitor emulsified in Freund's incomplete adjuvant were given intraperitoneally on week 4 and intramuscularly on weeks 6 and 8. The rabbits were bled on weeks 9 and 10, and finally exsanguinated on week 11. The antiserum was tested for monospecificity by double diffusion in agarose gels and immunoelectrophoresis (2.7) against normal human serum, and for specificity by using the same procedures against the purified PIP-inhibitor. Later antisera to the high molecular weight gp60 were produced using the same immunization schedule.

3.1.23 PREPARATION OF F(ab')₂ FRAGMENTS FROM RABBIT IgG

The IgG fraction of normal rabbit serum and the antiserum to the PIP-inhibitor were purified as described in 3.1.1. The IgG was dialysed against 0.1 M acetate buffer (pH 4.0). Porcine pepsin was added at an enzyme:substrate ratio of 1:100, and the mixture was incubated overnight at 37 °C. The digest was then buffered by the addition of solid Tris to pH 8.0, centrifuged (2000g for 15 min. at 2 °C), and the supernatant was dialysed against PBS. The F(ab')₂ fragments were separated from undigested IgG and pFc fragments by protein A-Sepharose chromatography using 0.5 x 3 cm column equilibrated in PBS. The exclusion peak was then collected and stored at -20 °C. The purity of the (Fab')₂ preparations was assessed by SDS-PAGE run under reducing and non-reducing conditions in 10% gels.

3.1.24 PREPARATION OF Fab FRAGMENTS FROM RABBIT IgG

IgG was purified from normal rabbit serum and the PIP-inhibitor antiserum as described in 3.1.1. Ten ml of rabbit IgG (1mg/ml) was dialysed against 0.5 M sodium phosphate buffer (pH 8.0), containing 2 mM EDTA and 10 mM cysteine. Papain was added to the IgG to give an enzyme:substrate ratio of 1:100, and the mixture was incubated at 37 °C for 18 hours. It was then centrifuged (2000 g for 10 minutes at 2 °C) and the supernatant was collected and dialysed against VBS at 4 °C for a further 18 hours, prior to application to a 3 ml column of protein A-Sepharose equilibrated in VBS. The column was then washed with VBS and the exclusion peak

which contained the Fab fragments was collected, while the undigested IgG and the Fc fragments remained bound to the column. After pooling, the exclusion peak was tested for purity by SDS-PAGE run under reducing and non-reducing conditions in 10% gels. The preparation was then stored in aliquots at -20 °C.

3.1.25 BIOTIN CONJUGATION OF IgG ANTI-PIP INHIBITOR

The IgG fraction of antiserum to the PIP-inhibitor was adjusted to a concentration of 5 mg/ml in PBS, and a biotin solution (11 mg/0.5 ml dimethylformamide) was added. The mixture was left to stand at room temperature for two hours, followed by dialysis against five changes of 2 l PBS for 18 hours to remove the unconjugated biotin. Finally the conjugate was stored at 4 °C with 0.01% thiomersal.

3.1.26 ENZYME-LINKED IMMUNOSORBENT ASSAY FOR PIP-INHIBITOR

The wells of high binding microtitre plates (Nunc) were coated with 1 µg/ml F(ab')₂ anti-PIP-inhibitor in 10 mM carbonate/bicarbonate buffer (pH 9.6, 100 µl/well) for 18 hours at 4 °C. The wells were washed five times with PBS-Tween (PBS containing 0.05% v/v Tween-20), and the unreacted sites blocked by incubation for 1 hour at room temperature with PBS-Tween containing BSA (0.1% w/v) (200 µl/well). The wells were then washed five times with PBS-Tween, and incubated with known concentrations of purified PIP-inhibitor, or test samples, for one hour at room temperature. After washing five times the wells were incubated with a 1:1000 dilution of biotin-anti-PIP-

inhibitor (100 μ l/well) for another one hour at room temperature. Following a further five washes, HRP-avidin at 1:1000 dilution (100 μ l/well) was added and the incubation continued for further hour at room temperature. Removal of unbound enzyme by washing five times in PBS-Tween was followed by the developing reaction in which 100 μ l of the developing reagent (25 ml 0.1 M citric acid and 25 ml 0.2 M di-sodium hydrogen phosphate, containing 17 μ l hydrogen peroxide and 17 μ g O-phenyldiamine, pH 5.4) was added to each well. The reaction was terminated by the addition of 25 μ l of 4 N sulphuric acid per well, and the colour intensity read using an automatic MR700 microplate ELISA reader at 492 nm (Dynatech Laboratories Ltd.).

3.1.27 SUCROSE DENSITY GRADIENT CENTRIFUGATION

Sucrose solutions were made up in VBS. Discontinuous gradients composed of 1 ml of 10%, 20%, 30%, and 40%, were formed on ice in 13 x 51 mm polyallomer centrifuge tubes. Five hundred microlitres (500 μ l) of the sample was layered carefully on the top of the gradient which was then centrifuged at 105,000 g for 16 hrs at 4^o C on a Beckman model L2-656 ultracentrifuge. Fractions of 100 μ l or 200 μ l were collected from the base of the tube using a sucrose gradient fractionator. The IgM, IgG, C1q, C3 and albumin content of each fraction was measured by single radial diffusion (2.6), the content of PIP-inhibitor by ELISA and radioactivity using an automatic gamma counter.

3.1.28 DETERMINATION OF THE TRUE MOLECULAR WEIGHT OF THE PIP-INHIBITOR

Five microgrammes (5 μ g) of PIP-inhibitor was electrophoresed on a series of different percentage (6, 7, 8, 9, 10, 11 and 12%) polyacrylamide gels (SDS-PAGE 2.8) run under reducing conditions. The molecular weight of the PIP-inhibitor was determined for each gel, and the true molecular weight was calculated by plotting percentage gel against the logarithm (\log_{10}) of the molecular weight and extrapolating the line to 0% gel concentration.

3.1.29 NEURAMINIDASE TREATMENT OF THE PIP-INHIBITOR

A mixture of 0.5 mg PIP-inhibitor in 50 mM sodium acetate buffer (pH 5.5), and 5 μ g neuraminidase, was incubated at 37 °C for one hour. The mixture was examined by immunoelectrophoresis (2.5) and by using SDS-PAGE (2.8) run under reducing conditions in 10% gels.

3.1.30 CHEMICAL DEGLYCOSYLATION OF THE PIP-INHIBITOR (Edge et al., 1981)

The PIP inhibitor was deglycosylated by the incubation of 0.5 ml trifluoromethylsulphonic anhydride (TFMS), containing 5% (v/v) anisole with 2 mg salt free lyophilised PIP-inhibitor in two stoppered conical glass tubes. The tubes were flushed with nitrogen, stoppered and sealed with parafilm. The reactants were incubated at 0 °C for 1 and 2 hours, after which times the reactions were terminated by the addition of anhydrous sodium carbonate, with vortexing to neutralise the acid. The reactants were then dialysed against water at 4 °C and the precipitated protein was

lyophilised. The deglycosylated protein was examined by SDS-PAGE (2.8) run under reducing conditions in 10% gels.

3.1.31 REDUCTION AND ALKYLATION OF ¹²⁵[I]-PIP INHIBITOR

Two hundred and fifty microgrammes (250 μ g) ¹²⁵[I]-PIP-inhibitor in 1 ml PBS, were incubated with 10 μ l of 0.1 M dithiothreitol for 30 minutes at room temperature, followed by the addition of 100 μ l ice-cold 0.015 M iodoacetamide. The reaction was allowed to proceed for 2 hours at 0 °C, after which the reduced and alkylated protein was dialysed against PBS at 4 °C and stored at -20 °C.

3.1.32 PROTEOLYTIC DIGESTION OF NATIVE AND REDUCED & ALKYLATED PIP-INHIBITOR

Native or reduced and alkylated ¹²⁵[I]-PIP-inhibitor were incubated with trypsin, chymotrypsin, V8 protease, and elastase in PBS at enzyme substrate ratio of 1:10, 1:100, 1:1000 for the native, or 1:10, 1:100, 1:1000, 1:10 000, and 1:100 000 for reduced and alkylated protein, at 37 °C for 1 and 24 hours. The digestions were terminated by the addition of Laemmli sample buffer (2.8), and analysed by SDS-PAGE (2.8) using 15% gels run under reducing conditions. The gels were then stained (2.9), dried, and the distribution of the radioactivity was analysed by autoradiography (Swanstrom & Shank, 1978).

RESULTS

3.2.1 PURIFICATION OF THE PIP-INHIBITOR (METHOD 1)

During IgG-Sepharose chromatography of normal human serum, the PIP-inhibitory material was eluted from the column as a single peak, along with other IgG-binding proteins (Fig. 3.1), using 2 M guanidine-hydrochloride, and was not found elsewhere in the chromatogram. Previous attempts to elute the PIP-inhibitor with low pH buffer (glycine-HCl pH 3.0) succeeded in only eluting a small proportion of the PIP-inhibitor from the column. The PIP-inhibitory material from the IgG-Sepharose column (50 ml) had a total protein concentration of 0.91 mg/ml and produced 50 % inhibition of PIP.

Protein A-Sepharose chromatography of this pool (Fig. 3.2) resulted in most of the PIP inhibitory material being excluded from the column, while IgG, the main contaminant was bound. The exclusion peak, contained 280 µg/ml of protein and showed 42% inhibition of PIP. This peak (64 ml) was applied to Con.A-Sepharose affinity chromatography (Fig. 3.3), which resulted in the PIP-inhibitor being confined to the first 40 fractions, which produced 25% inhibition of PIP.

On SDS-PAGE run under reducing and non-reducing conditions, the purified PIP-inhibitor showed a single band with a molecular weight of 60 kD (Fig. 3.4). The preparation also gave a single precipitin line when analysed against anti-whole human serum by double diffusion in agarose gel (Fig. 3.15) and immunoelectrophoresis (Fig. 3.16).

Figure 3.1:

IgG-Sepharose chromatography of normal serum. Total protein (—) measured at 280 nm, inhibition of PIP (●--●), conductivity (Δ—Δ). The points of application of 150, 500 mM NaCl and 2M guanidine hydrochloride are shown by the arrows.

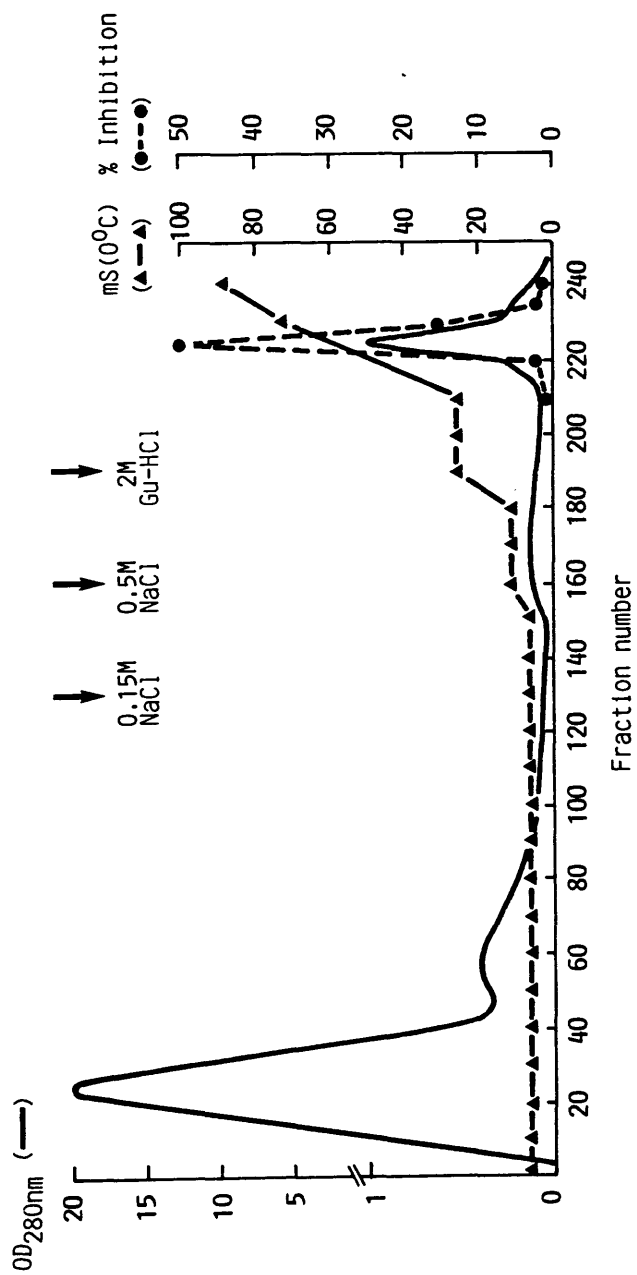


Figure 3.2:

Protein A-Sepharose affinity chromatography
of PIP-inhibitory peak from IgG-Sepharose column.
Total protein was measured at 280 nm (—),
inhibition of PIP (•—•) measured by the described
assay in 3.1.5.

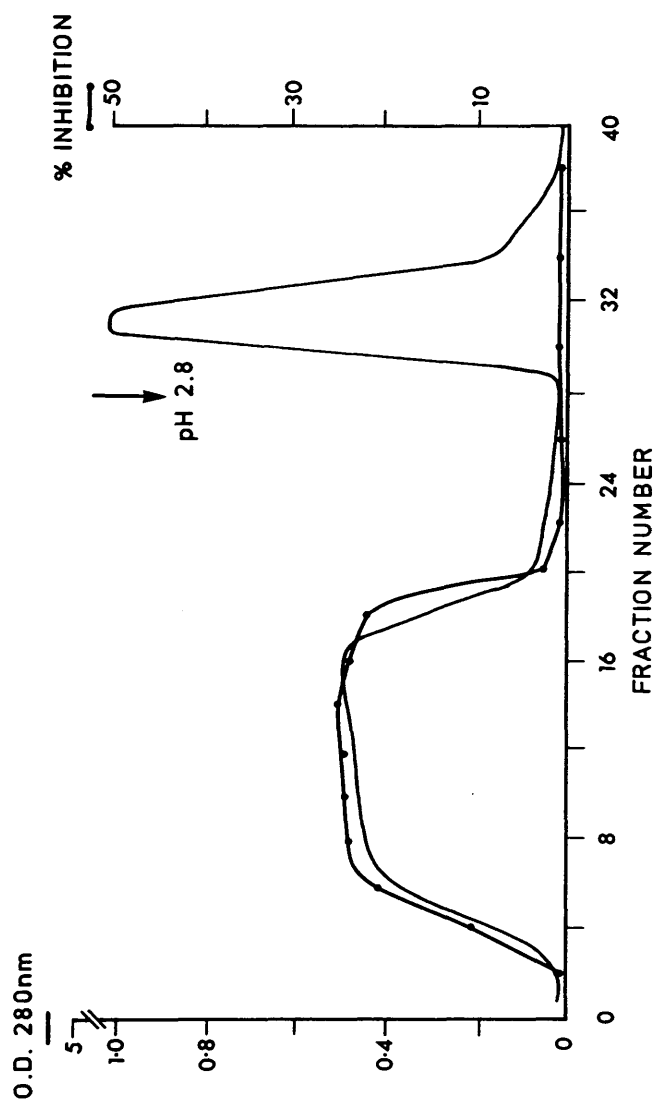


Figure 3.3:

Con.A-Sepharose affinity
chromatography of exclusion peak of
protein A-Sepharose column. Total
protein measured by absorbance at
280 nm(—), inhibition of PIP
(●—●) by the described assay.

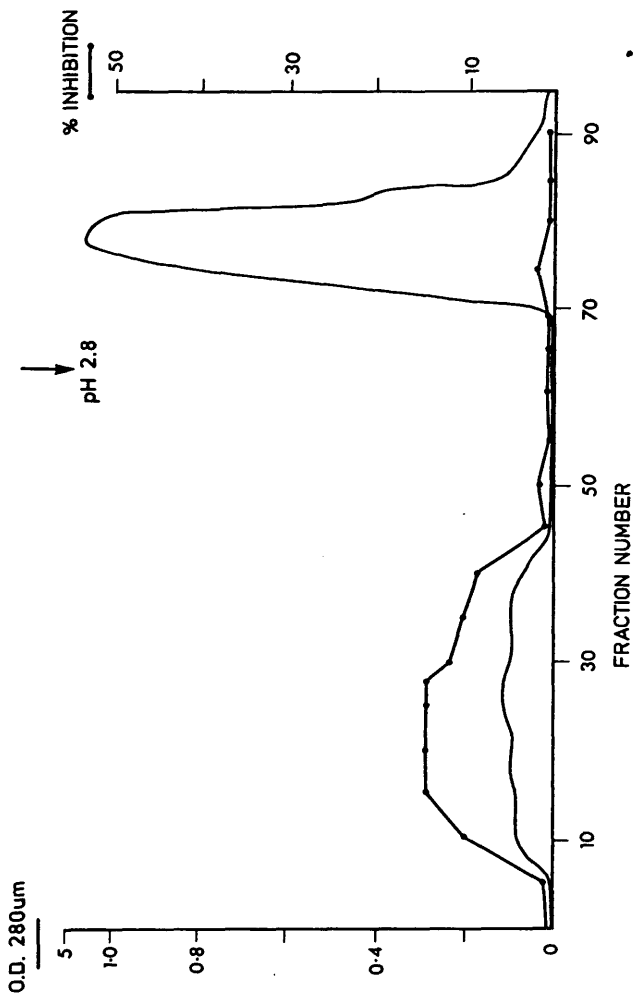


Figure 3.4:

Silver stained 10% SDS-PAGE. Lanes 1-7 run under reducing conditions, lanes 8 and 9 run under non-reducing conditions. Lanes were loaded as follows: (1) molecular weight markers; (2) normal human serum; (3) PIP inhibitory peak from IgG-Sepharose; (4) exclusion peak of protein A-Sepharose; (5) material bound to protein A-Sepharose; (6) exclusion peak of Con.A-Sepharose; (7) bound material to Con.A-Sepharose; (8) molecular weight markers; (9) exclusion peak of Con.A-Sepharose. The single band in lanes 6 and 9 has an apparent molecular weight of 60 kD.

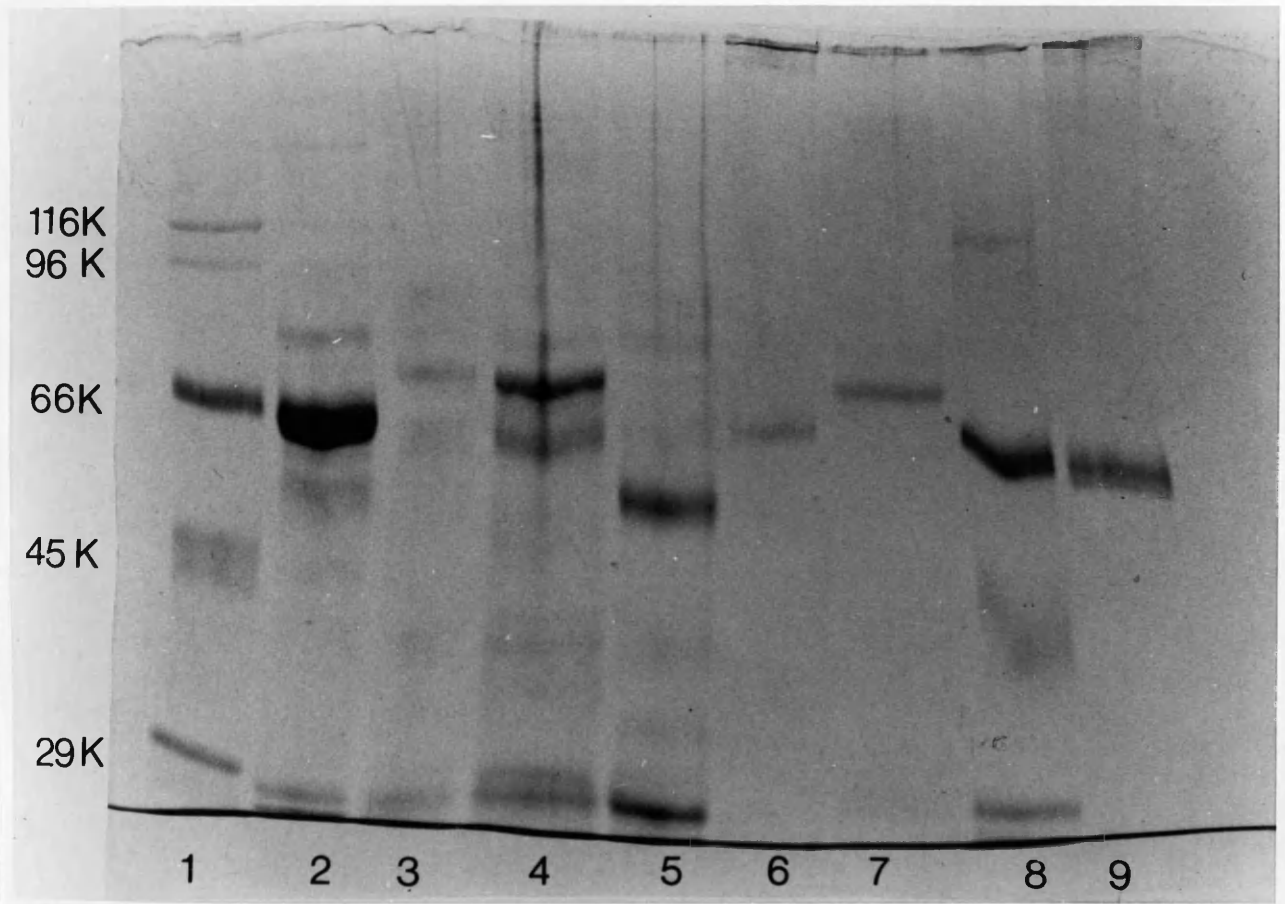


Figure 3.5:

7.5% SDS-PAGE of purified gp60

**(prepared by method 1)(lane 1) and normal human
serum (lane 2) stained with PAS.**

66

1

2

As the PIP-inhibitor reacted positively in the PAS reaction (Fig. 3.5), it was considered to be glycoprotein. It was therefore called glycoprotein 60 (gp60), and this name will be used throughout the remainder of this thesis.

A purification summary of gp60 prepared by Method 1 is presented in Table 3.1. Using an ELISA procedure (3.1.26), the concentration of gp60 was measured in the starting material and in pools prepared at different stages of the purification procedure. It can be seen that the starting material contained 1.6 mg of gp60 (32 $\mu\text{g}/\text{ml}$), while the purified protein preparation contained 783 μg , a yield of 49%, and the degree of purification was 1518-fold.

During subsequent investigations aimed at the characterisation of gp60, sucrose density gradient and gel-filtration chromatography (Sephacryl-S300) studies of purified gp60 and gp60 in whole human serum were performed. It was found that in normal human serum the majority of the gp60 sedimented immediately along with IgM (19S) in sucrose density gradient centrifugation and was eluted in a similar position to IgM on gel filtration chromatography. A minor proportion (approximately 5-10%) appeared to be of low molecular weight (see section 3.2.5.). The purified gp60 had the properties of the low molecular weight material on sucrose density gradient centrifugation and Sephacryl-S300 gel filtration chromatography. The possibility that the low molecular weight material represented proteolytically cleaved gp60 was considered, so it was decided to repeat the gp60 preparation using protease inhibitors throughout the purification procedures.

TABLE 3.1 PURIFICATION SUMMARY FOR gp60 PREPARED BY METHOD 1

	Volume (ml)	Total protein conc. (mg/ml)	gp60 conc. (μg/ml)	Total amount of protein (mg)	Total amount of gp60 (mg)	Yield gp60 (%)	$\frac{\text{Total gp60 } (\mu\text{g})}{\text{Total protein}} (\mu\text{g})$	Degree of purification
Serum	50	60.1	31.7	3005	1.6	100	0.0005	-
IgG-Sepharase	50	0.91	23	45.5	1.2	75	0.026	51
Protein A-Sepharase	64	0.28	17	17.9	1.1	69	0.062	123
Con. A-Sepharase	77	0.008	10	0.584	0.77	49	0.759	1518

3.2.2 PURIFICATION OF gp60 (METHOD 2)

Method 1 of the gp60 purification procedure was scaled up to increase the yield of gp60 by using 500 ml of normal serum. As in method one, gp60 was eluted from IgG-Sepharose as a single peak (Fig. 3.6), which contained 1.6 mg/ml total protein, 96 μ g/ml gp60 and showed 96% inhibition of PIP.

IgG contaminants were removed by protein A-Sepharose chromatography, while most of the gp60 was confined to the exclusion peak, which contained 0.64 mg/ml total protein, 71 μ g/ml gp60 and showed 85% inhibition of PIP. This pool (160 ml) was concentrated by ultrafiltration to 10 ml which was divided into two 5 ml aliquots which were applied separately to a Sephacryl-S300 gel filtration column. Gp60 co-eluted with IgM, C4bp and fibronectin in the first protein peak (Fig. 3.7). The combined gp60 pools from the two chromatograms contained 1.7 mg/ml total protein, 331 μ g/ml gp60 and produced 97% inhibition of PIP. This pool was subjected to heparin-Sepharose affinity chromatography, which successfully removed the major contaminants, IgM, C4BP and fibronectin which were bound to the column while gp60 appeared in the exclusion peak (Fig. 3.8.). This pooled exclusion peak contained 8.2 mg gp60 and inhibited PIP by 95%. The preparation gave a single band of 60 kD when analysed by SDS-PAGE run under reducing and non-reducing conditions in 10% gels (Fig. 3.9.), and a single precipitin line against anti-whole human serum on double diffusion in agarose gel (Fig. 3.15) and on immunoelectrophoresis (Fig. 3.16).

Figure 3.6:
IgG-Sepharose affinity chromatography
of normal serum (method 2). Total protein
measured by absorbance at 280 nm (●—●),
conductivity (Δ---Δ), gp60 concentration
measured by ELISA (○—○).

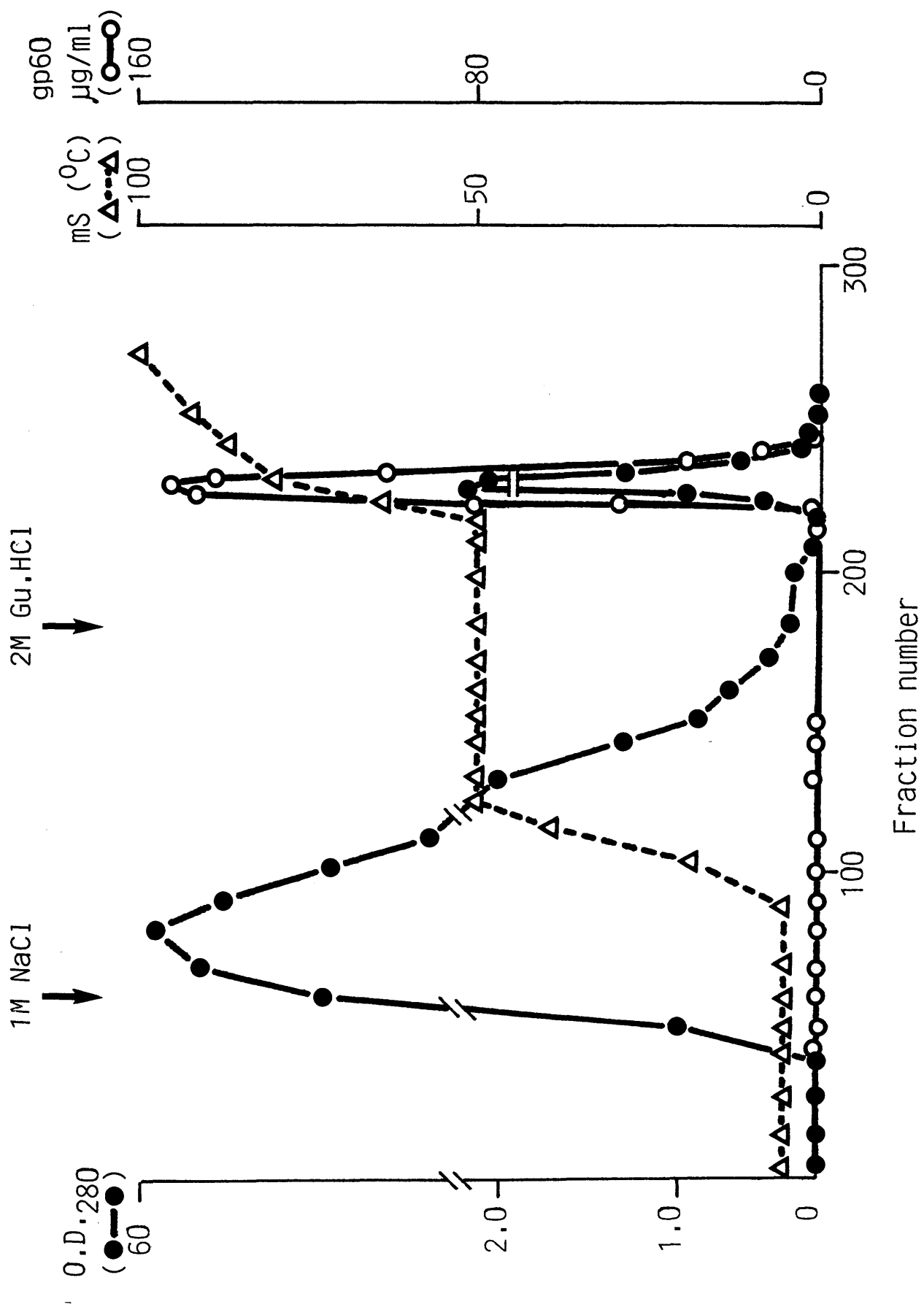


Figure 3.7:

Sephacryl-S300 gel filtration of the
exclusion peak of protein A-Sepharose.

Total protein (—) measured at

O.D. and gp60 levels (O — O) measured by ELISA.
280

The arrows show the elution volume for IgM, C1q,
IgG and albumin.

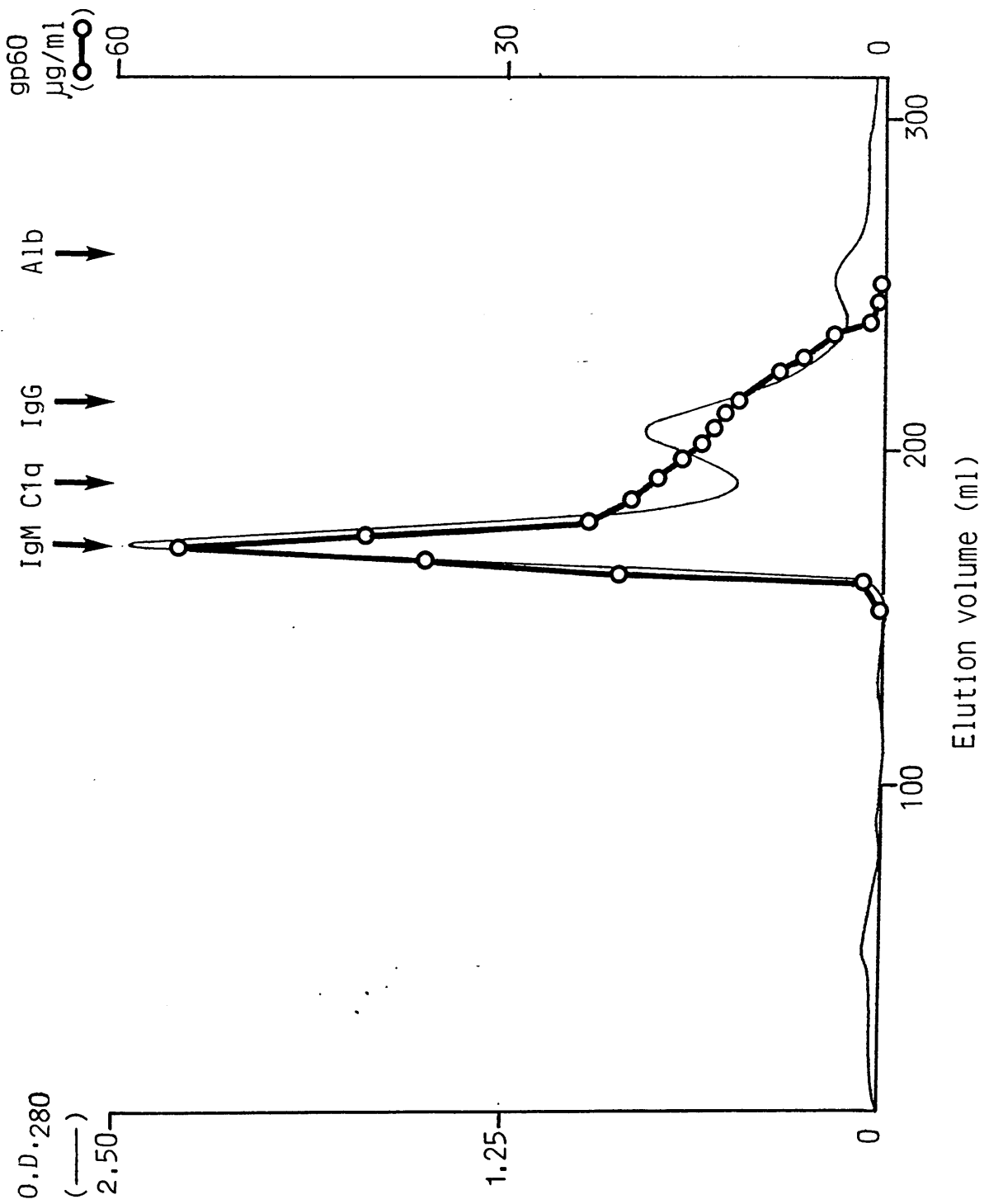


Figure 3.8: Heparin-Sepharose chromatography of Sephacryl-S300 gp60 pool. Total protein (●-●) was measured by BCA assay, conductivity (▲--▲) at 0 °C and gp60 by ELISA. The arrow shows the application point of the gradient which consisted of 300 ml starting buffer and 300 ml starting buffer containing 0.75 M NaCl.

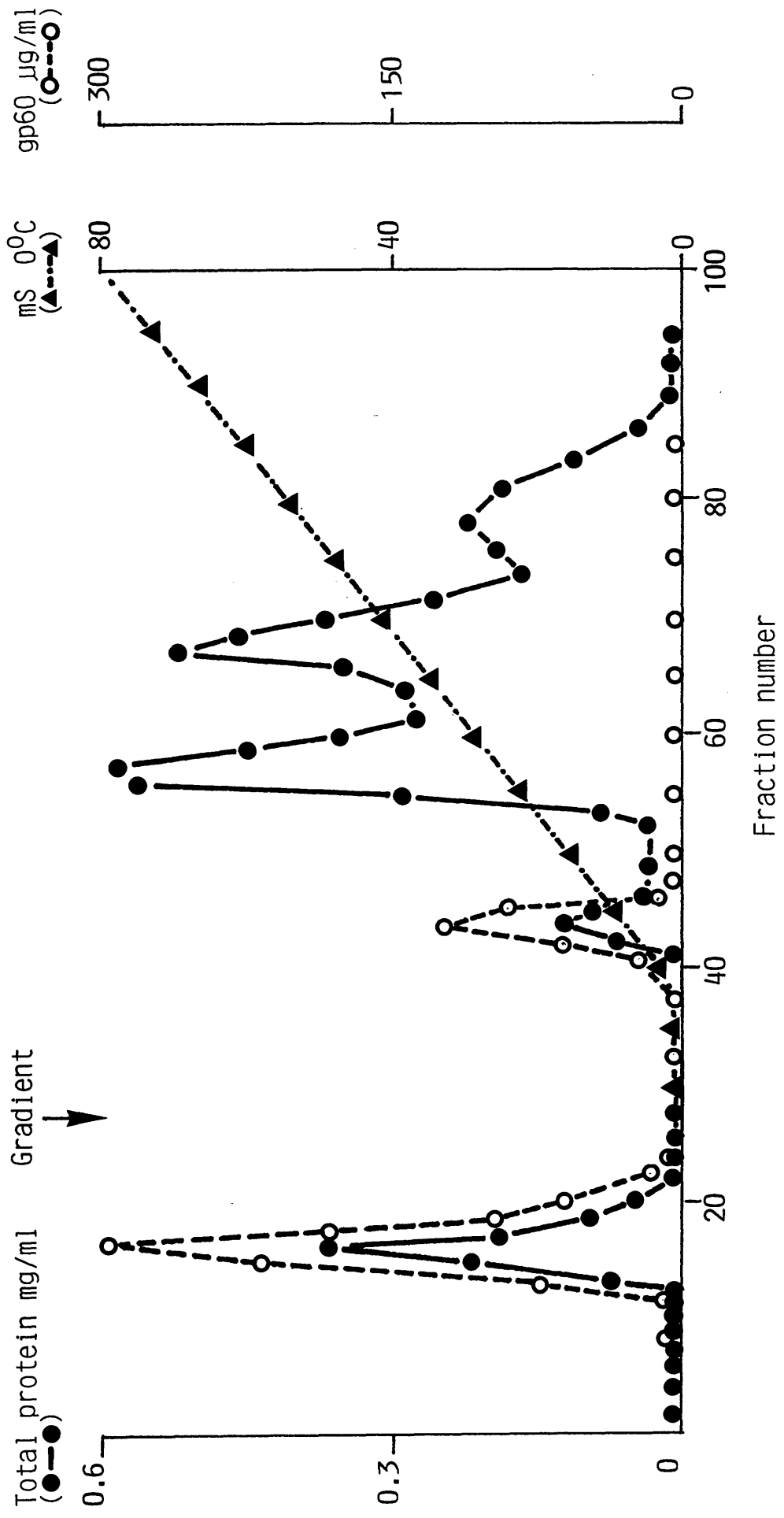


Figure 3.9:

10% SDS-PAGE for gp60 purified by method 2. Lanes 1 and 2 run under reducing conditions, lanes 3 and 4 run under non-reducing conditions. Lanes 1 and 3 were loaded with molecular weight markers, and lanes 2 and 4 were loaded with gp60 purified by method 2.

A purification summary for the purification of gp60 by Method 2 is presented in Table 3.2, where the starting material contained 16 mg of gp60 (32 μ g/ml), while the purified preparation contained 8.2 mg, representing a yield of 51% and a degree of purification of 1956-fold.

3.2.3 PURIFICATION OF gp60 (METHOD 3)

As it was proposed to study the functional properties of gp60 particularly with respect to its binding to IgG and inhibition of PIP, the possibility was considered that the harsh conditions required for the elution of gp60 from IgG-Sepharose might reduce its functional activity. It was therefore decided to purify gp60 from whole serum using a procedure which avoided the initial step of binding to IgG-Sepharose.

During the DEAE-Sephacel anion exchange chromatography (Fig. 3.¹⁰₁₀), most of the IgG was eliminated in the exclusion peak. Gp60 was eluted from the column with a linear salt gradient at a conductivity of 8-16 mS (at 0^o C). The gp60 pool was applied to Biorex-70 cation exchange chromatography (Fig. 3. 11), which resulted in the removal of most of the proteins in the exclusion peak, while gp60 was eluted by a linear salt gradient.

This pool was concentrated and subjected to gel filtration (Sephacryl-S300), which resulted in the co-elution of gp60 with IgM, C4BP and fibronectin in first protein peak (Fig. 3. 12). This peak inhibited PIP by 89%, and contained 0.92 mg/ml total protein and 195 μ g/ml gp60. On the application of gp60 pool to heparin-Sepharose, all

TABLE 3.2 PURIFICATION SUMMARY FOR gp60 PREPARED BY METHOD 2

	Volume (ml)	Total protein conc. (mg/ml)	gp60 conc. (µg/ml)	Total amount of protein (mg)	Total amount of gp60 (mg)	Yield gp60 (%)	$\frac{\text{Total gp60 (µg)}}{\text{Total protein (µg)}}$	Degree of purification
Serum	500	64.3	32.0	32150	16	100	0.0005	-
IgG-Sepharose	160	1.6	96	256	15.4	96	0.06	120
Protein A-Sepharose	160	0.64	471	102.4	11.4	71	0.111	222
Gel Filtration	25	1.68	331	42	8.3	52	0.198	396
Heparin-Sepharose	40	0.21	204	8.4	8.2	51	0.976	1952

Figure 3.10:

DEAE-Sephacel anion exchange chromatography of normal serum. Total protein (●) measured by BCA assay, conductivity (▲), gp60 (○) by ELISA. The arrow shows the application point of the gradient which consisted of 5l starting buffer and 5l starting buffer containing 0.5M NaCl. Gp60 was eluted at 8 mS.

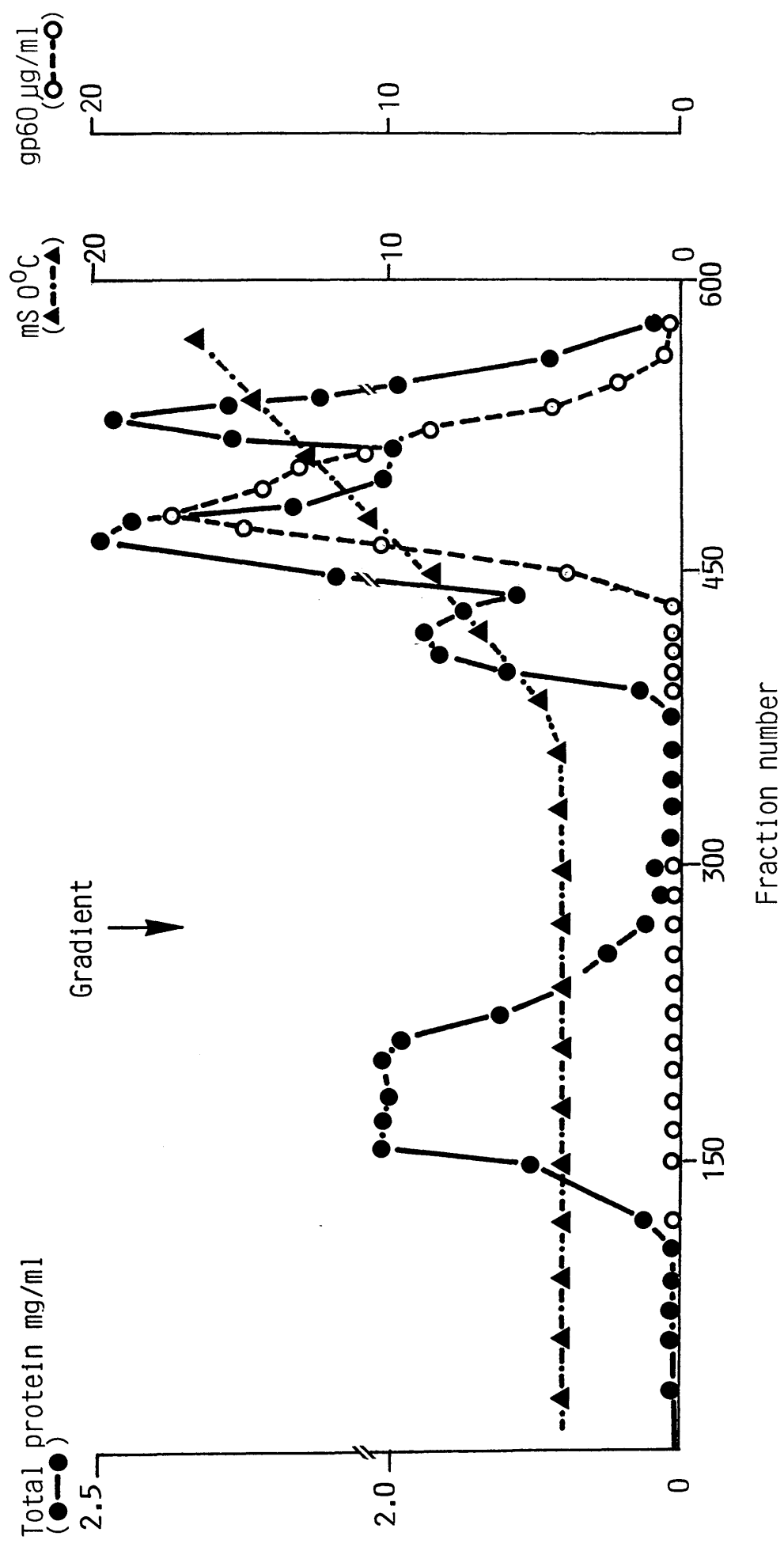


Figure 3.11:

Biorex-70 cation exchange chromatography of gp60 pool from DEAE-Sephacel column. Total protein (●—●), conductivity (▲---▲), gp60 (○--○) measured by ELISA. Gp60 was eluted with a gradient of 1l starting buffer and 1l starting buffer containing 0.7 M NaCl.

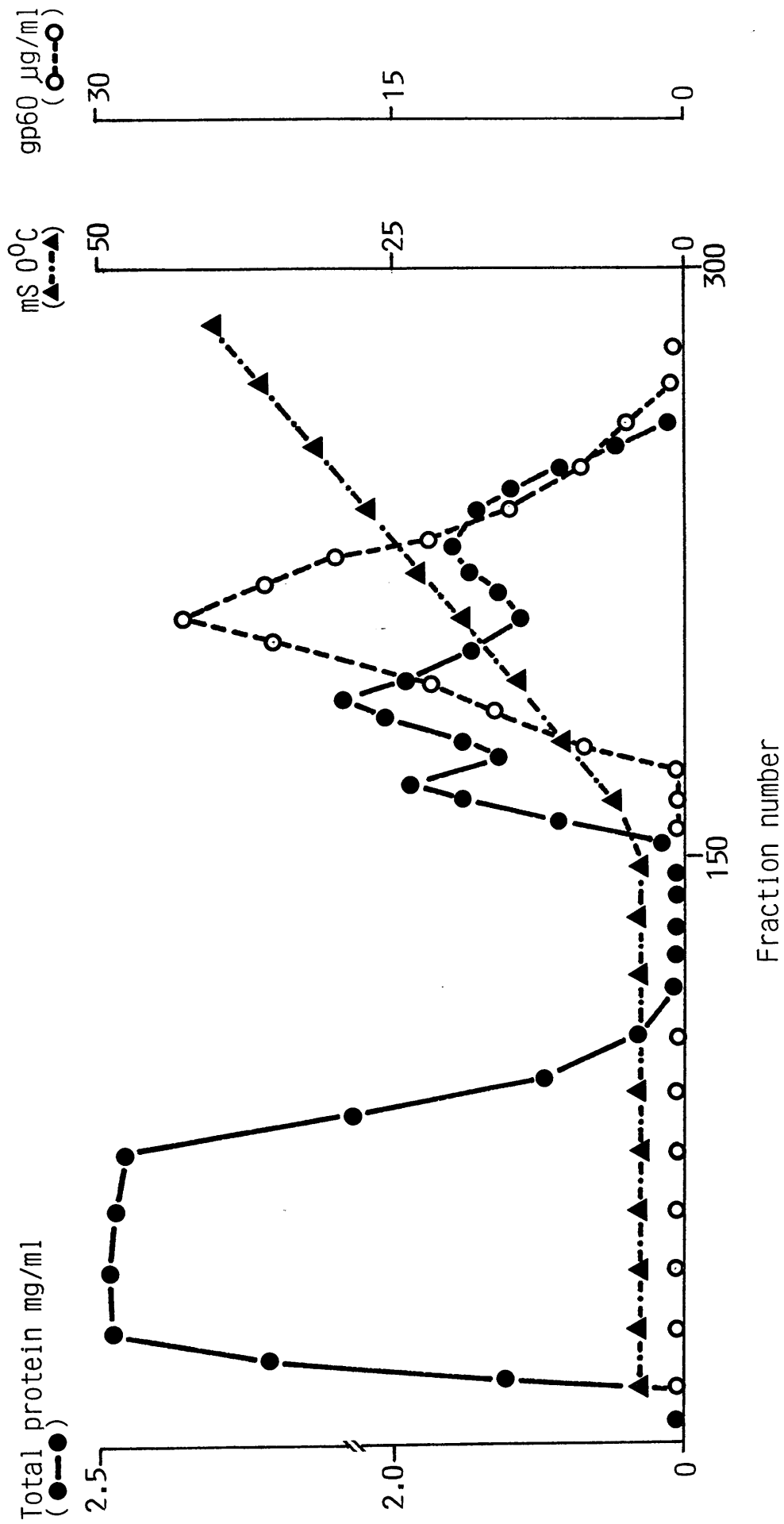
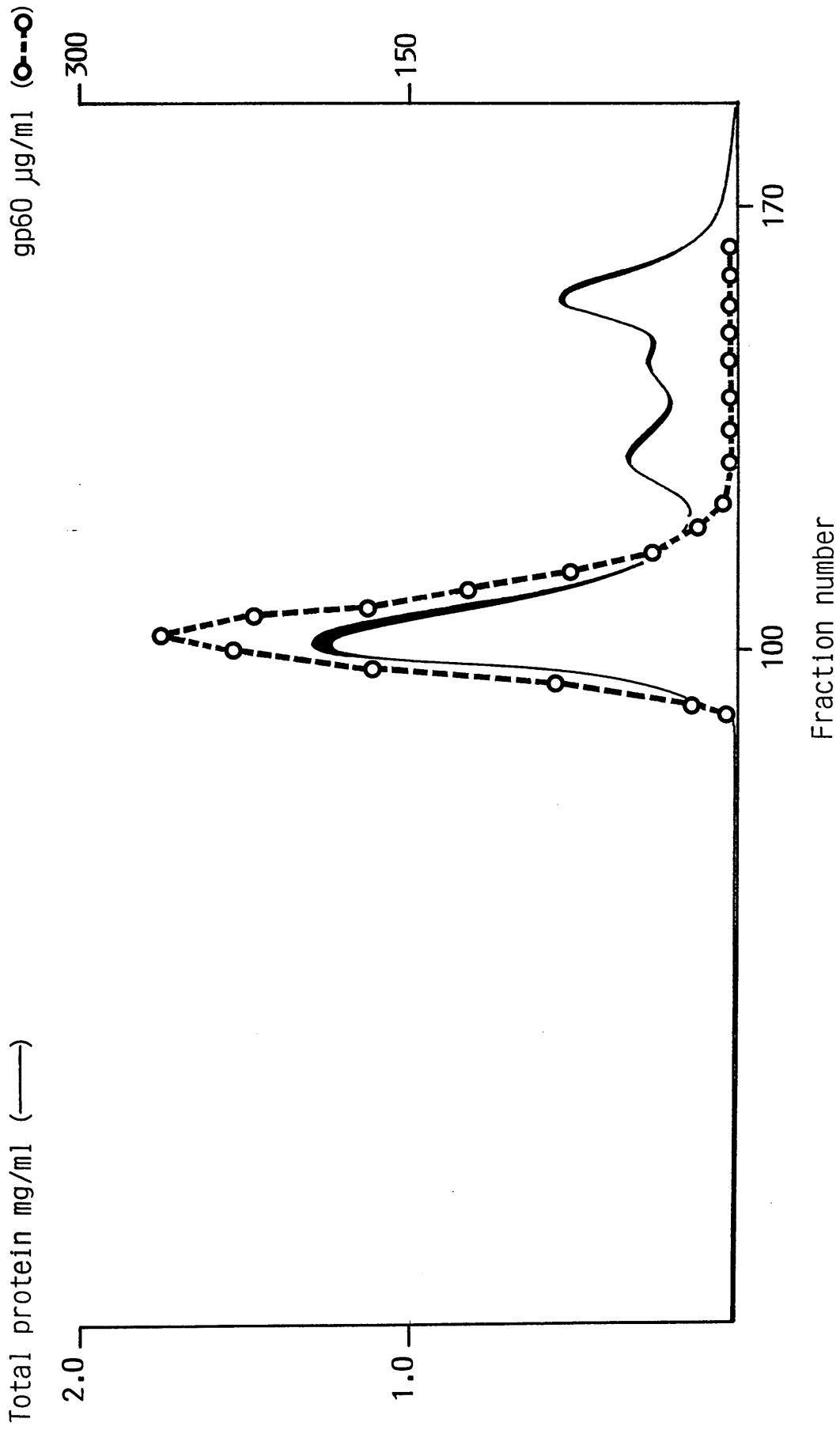


Figure 3.12:

Sephacryl-S300 gel filtration
chromatography of gp60 peak from Biorex-70
column. Total protein measured by BCA assay
(—), gp60 by ELISA (O--O).



of the contaminants were bound to the column, while gp60 (PIP inhibitory) activity was confined in the exclusion peak (Fig. 3.13). This preparation was homogeneous when analysed by SDS-PAGE run under reducing and non-reducing conditions in a 7.5% gel, which showed a single band with a molecular weight of 60 kD (Fig. 3.14).

The preparation was also tested against anti-whole human serum by double diffusion analysis in agarose gels, which resulted in the appearance of a single precipitin line (Fig. 3.15).

A purification summary for gp60 prepared by method 3 is presented in Table 3.3. The starting material contained 19 mg gp60 (19 μ g/ml), while the pure preparation contained 7.2 mg gp60 representing a yield of 38 % and a degree of purification 3360-fold.

3.2.4 CHARACTERISATION OF gp60

The protein concentration of gp60 preparations were determined by O.D₂₈₀, Folin assay and BCA assay (2.4). Both O.D₂₈₀ and Folin assay results agreed with each other, while the BCA assay results gave a value which was one sixth of the former two. The OD₂₈₀ and Folin assays measure the content of aromatic amino acids, particularly tyrosine and tryptophan, while the BSA assay, a modified Biuret technique, measures peptide bonds. This discrepancy suggested that gp60 possesses a high content of aromatic amino acids, which has been confirmed by Mr. Tony Willis (MRC Immunochemistry Unit, Oxford).

Figure 3.13:

Heparin-Sepharose chromatography of gp60 peak
from Sephacryl-S300 column.

Total protein (●-●) measured by BCA assay,
gp60 (○--○) by ELISA. The bound proteins
were eluted with 1M NaCl.

Total protein mg/ml
(●—●)

gp60 μ g/ml
(○- -○)

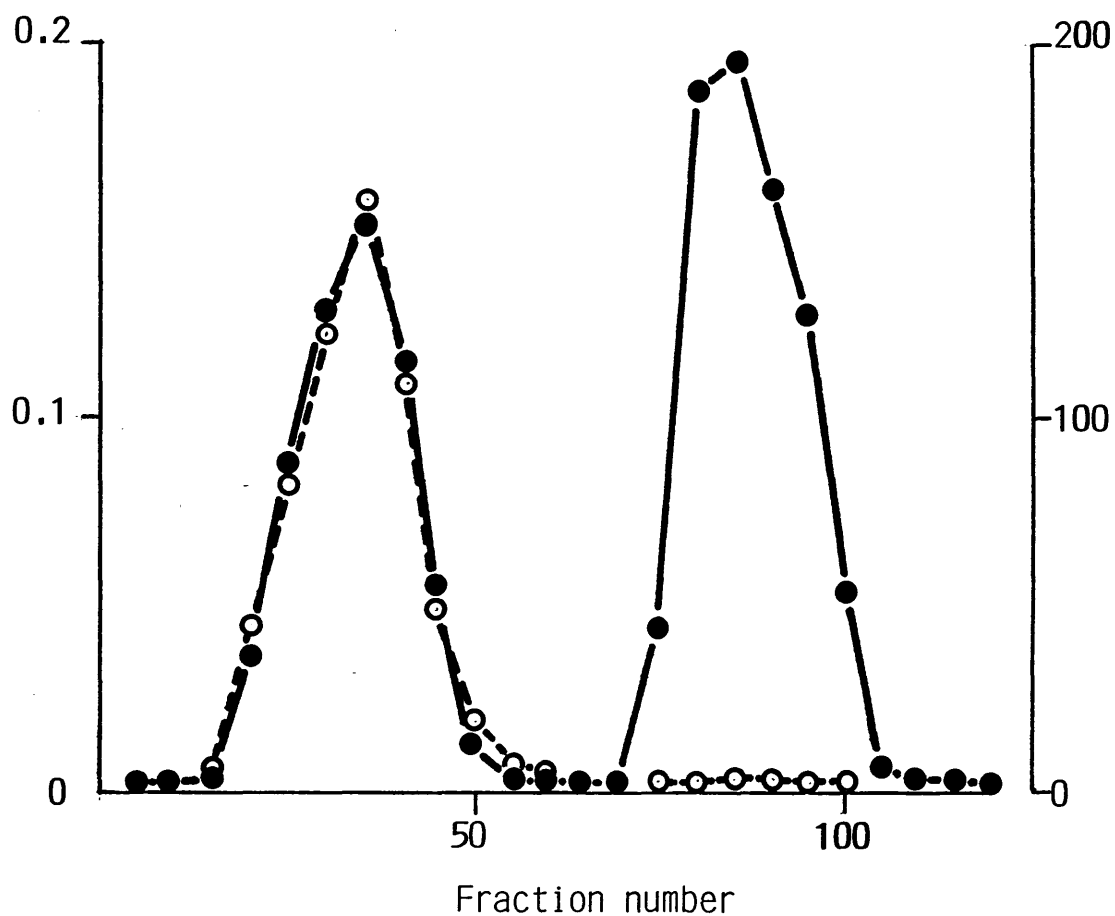


Figure 3.14:

Silver stained 7.5% SDS-PAGE, lanes 1 and 2 run under reducing conditions, lanes 3 and 4 run under non-reducing conditions. Lanes 1 and 3 were loaded with molecular weight marker, and lanes 2 and 4 were loaded with gp60 purified by method 3.

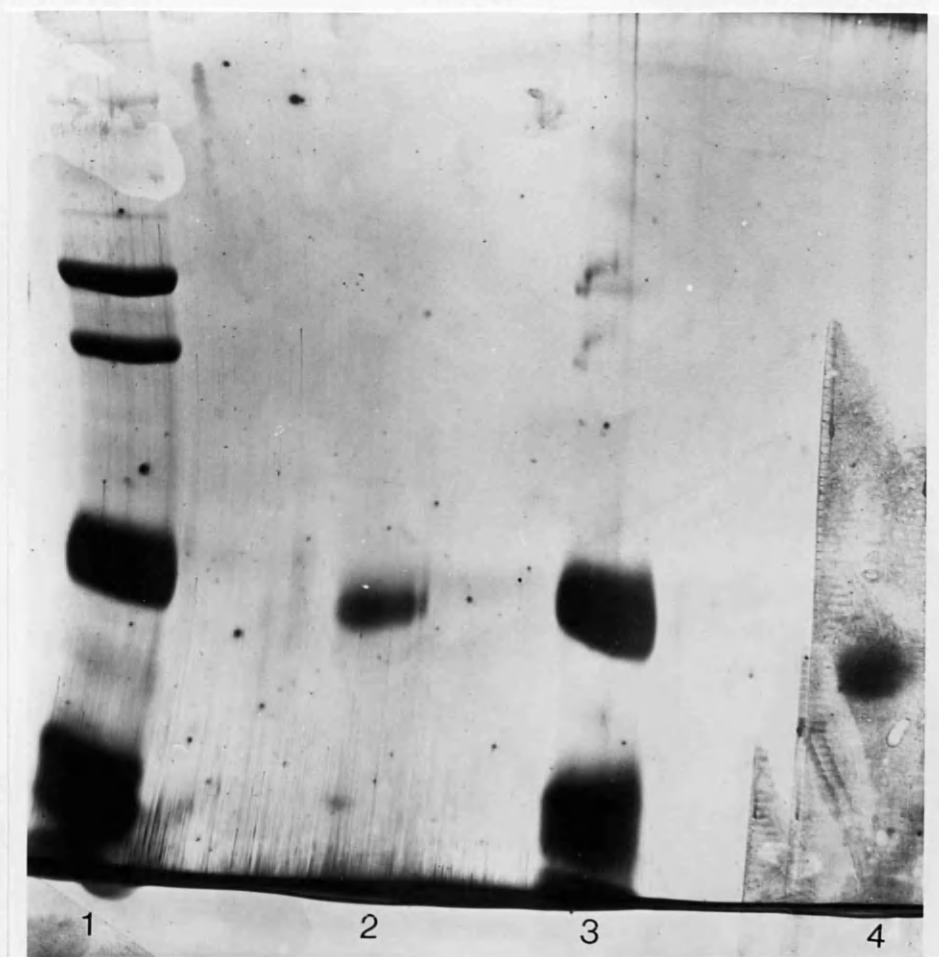


Figure 3.15:

Double diffusion analysis in agarose gel of gp60 purified by method 1 (well 1), gp60 purified by method 2 (well 2), gp60 purified by method 3 (well 3) and normal human serum (well 4). Center well (5) contained antiserum to whole human serum. Anti-normal human serum produced a line of complete identity between the three gp60 preparations and normal serum.

TABLE 1.3 PURIFICATION SUMMARY FOR GP60 PREPARED BY METHOD 3

	Volume (ml)	Total protein conc. (mg/ml)	Total amount of GP60 (mg)	Yield GP60 (%)	Total GP60 (ml) total protein	Degrees of purification
Serum	1000	59.4	19	100	0.0003	7
DEAE-Sephacel	1500	21.2	14.4	36	0.0005	2
Biolar-70	1200	1.4	8.2	48	0.0055	18
acrylamide-8300	40	4.52	7.5	41	0.212	207
Heparin-Sepharose	60	0.12	7.2	38	1.01	3363

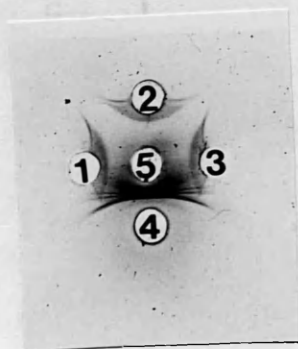


TABLE 3.3 PURIFICATION SUMMARY FOR gp60 PREPARED BY METHOD 3

	Volume (ml)	Total protein conc. (mg/ml)	gp60 conc. (μg/ml)	Total amount of protein (mg)	Total amount of gp60 (mg)	Yield gp60 (%)	$\frac{\text{Total gp60 } (\mu\text{g})}{\text{Total protein}} (\mu\text{g})$	Degree of purification
Serum	1000	59.4	19	59400	19	100	0.0003	-
DEAE-Sephacel	1500	21.2	9.6	31800	14.4	76	0.0005	2
Biotex-70	1200	1.4	7.7	1680	9.2	48	0.0055	18
Sephacryl-S300	40	0.92	195	36.8	7.8	41	0.212	707
Heparin-Sepharose	60	0.12	120	7.14	7.2	38	1.01	3360

On SDS-PAGE, the three preparations showed a single band with a molecular weight of 60kD when run under reducing and non-reducing conditions. Double diffusion analysis of the three preparations of gp60 against anti-whole human serum resulted in single precipitin lines with gave reactions of complete identity with each other. (Fig. 3.15).

On immunoelectrophoresis gp60 prepared by method 1 migrated as a prealbumin when tested against anti-whole human serum while gp60 prepared by methods 2 and 3 gave a single arc in the β / γ region (Fig. 3.16).

3.2.5. SUCROSE DENSITY GRADIENT CENTRIFUGATION AND GEL FILTRATION STUDIES

On sucrose density gradient of normal serum, it was found that the majority of gp60 had a 19 S sedimentation coefficient while approximately 5-10% sedimented with a coefficient of 2.9S (Fig. 3.18). Fractionation of normal serum on gel filtration (Sephacryl-S300) showed that most of gp60 which was present had a molecular weight of 1000 kD, while a minor proportion (approximately 5-10%) eluted from the column with a molecular weight of 58 kD. Gp60 (prepared by method 1) sedimented at 2.9S and on gel filtration chromatography it eluted with an apparent molecular weight of 58 kD. Gp60 prepared by methods 2 and 3 showed a sedimentation coefficient of 19S and molecular weight of 1000 kD on sucrose density gradient and gel filtration chromatography respectively (Figs. 3.18; 3.19).

Figure 3.16:

Immuno-electrophoresis of normal serum (wells 1 and 5), purified gp60 polymer prepared by method 2 (well 4), purified gp60 monomer prepared by method 1 (wells 2,3). Antiserum to whole human serum is in troughs a and d, and anti-gp60 is in troughs b, c and e. Anode to the left.

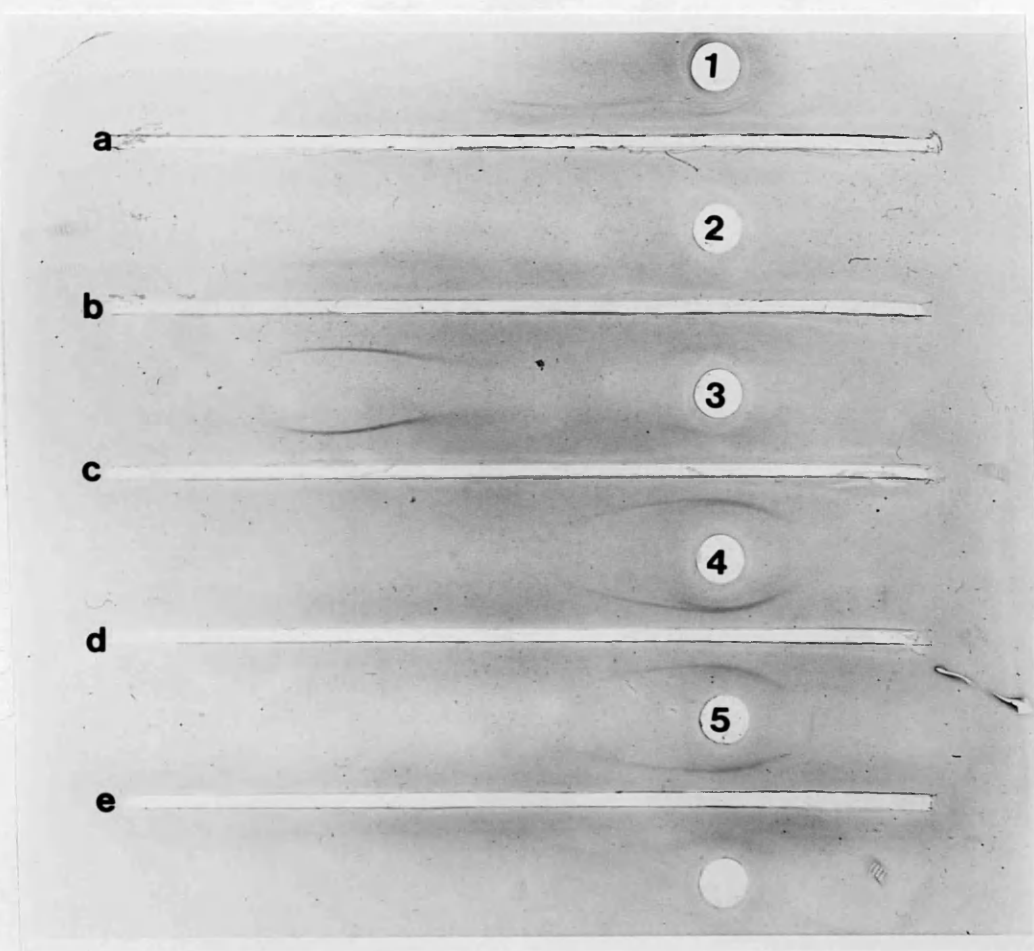


Figure 3.17:

Immunelectrophoresis of normal serum (well 1) and purified gp60 polymer prepared by method 3 (well 2). Antiserum to whole human serum is in troughs a and c and anti-gp60 in trough b. Anode is to the left.

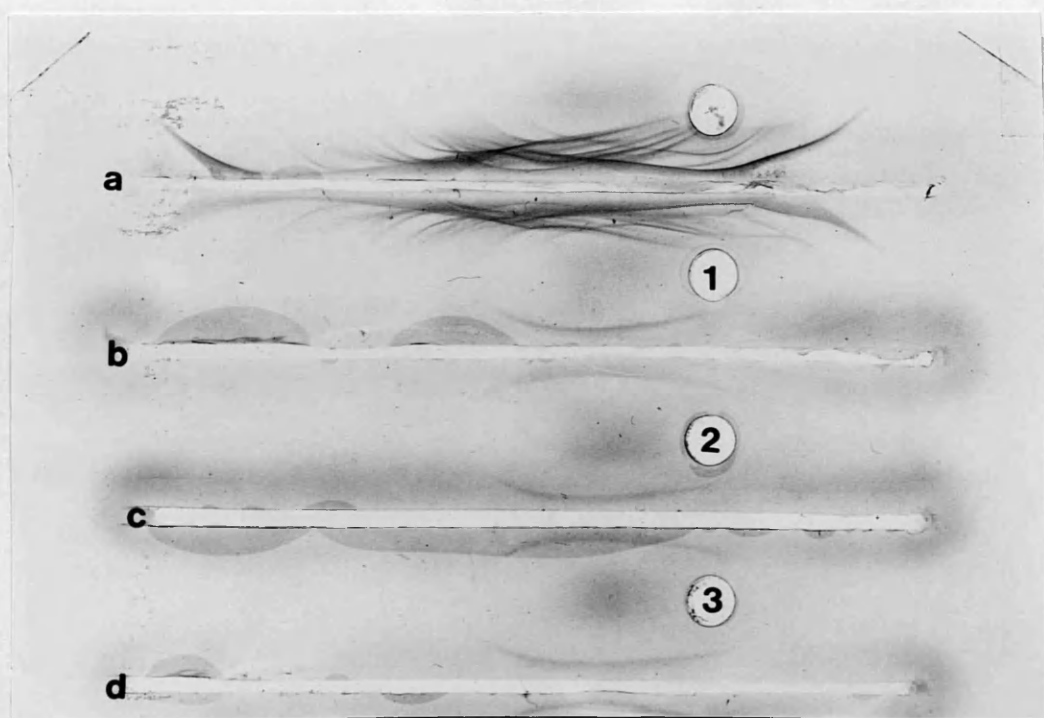


Figure 3.18:

Sucrose density gradient centrifugation of normal serum (●-●), gp60 prepared by method 1 (Δ-Δ) and gp60 prepared by method 2 (▲-▲). Gp60 concentrations in 250 ul fractions were measured by ELISA (3.1.26), while concentrations of IgM, C1q, C3, IgG and albumin were measured by single radial immuno-diffusion.

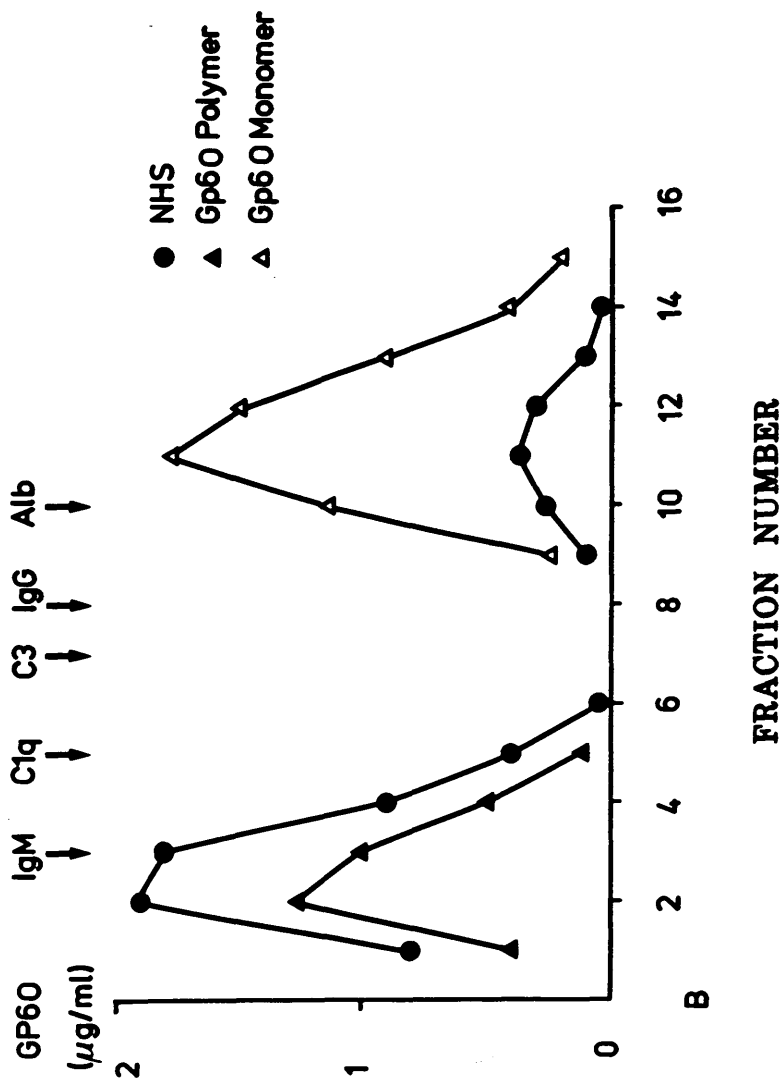
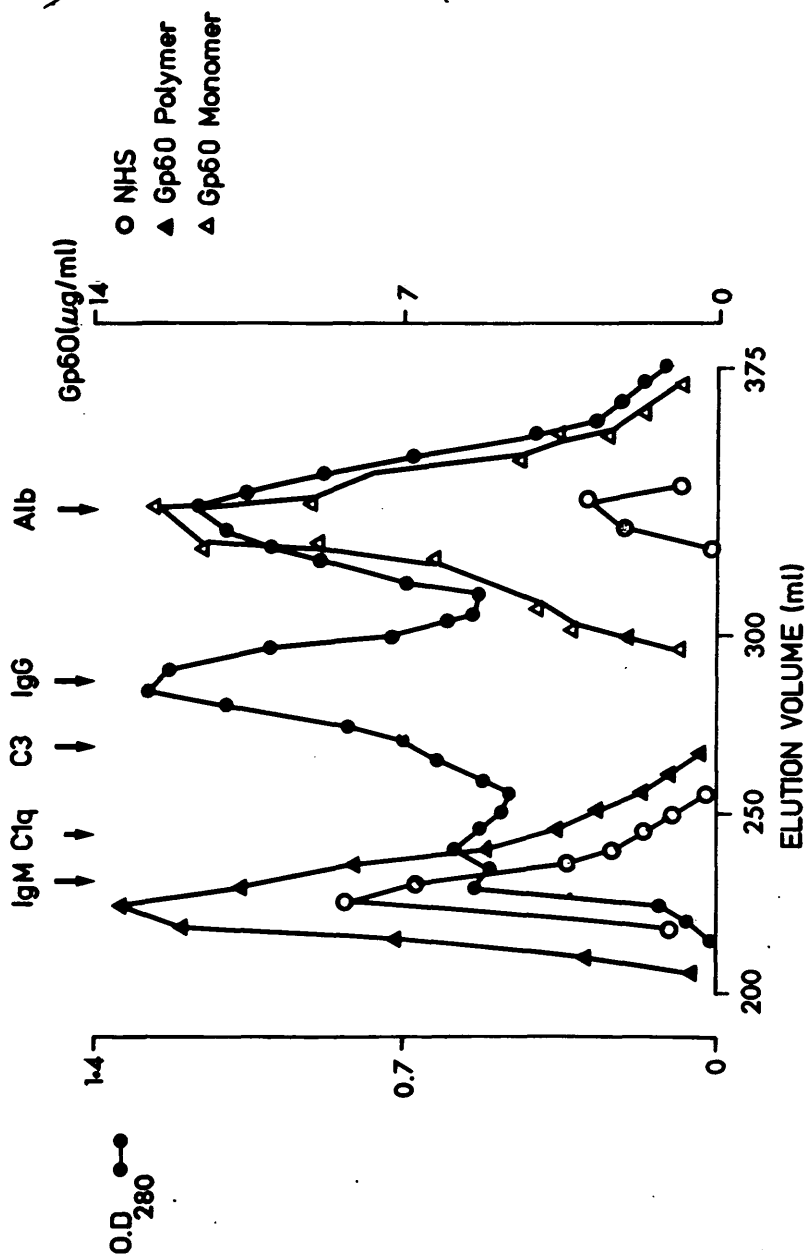


Figure 3.19:

Distribution of gp60 on Sephacryl-S300 gel
filtration of normal serum (O—O), gp60 prepared
by method 2 (▲—▲) and gp60 prepared by method 1
(△—△). Total protein (●—●) was measured by
O.D. nm and gp60 levels were measured by ELISA.



3.2.6 STUDIES OF gp60 AS A GLYCOPROTEIN

Gp60 (prepared by method 3) was sensitive to neuraminidase treatment. The neuraminidase-treated gp60 showed a reduction in its molecular weight from 60 to 52 kD when tested by SDS-PAGE run under reducing conditions, (Fig. 3.20). It also showed a shift in its electrophoretic mobility from B2/ γ to γ . These findings indicate that gp60 contains significant amounts of sialic acid (Fig. 3.21).

On chemical deglycosylation by TFMS, gp60 showed a reduction in its molecular weight from 60 kD to 48 kD when tested by SDS-PAGE under reducing conditions (Fig. 3.20).

The determination of the true molecular weight of gp60 from different percentage gels (Fig. 3.22), showed a true molecular weight of 43 kD. Thus, 28% of the weight of gp60 is carbohydrate.

3.2.7 LIMITED PROTEOLYSIS OF gp60

Gp60 was relatively resistant to proteolysis by trypsin (Fig. 3.3), chymotrypsin (Fig. 3.24), V8 protease (Fig. 3.25) and elastase (Fig. 3.26). Cleavage only occurred at high enzyme: protein ratio (1:10, 1:100) and required relatively long incubation periods (1-24 hours). Reduction and alkylation of the molecule did not seem to affect its proteolytic cleavage to a great extent (Figs. 3.27; 3.28; 3.29, 3.30).

Figure 3.20:

Comassie-blue stained 10% SDS-PAGE run under reducing conditions. Lanes were loaded as follows:-

Lane 1: Molecular weight markers.

Lane 2: Gp60 prepared by method 2.

Lane 3: Neuraminidase treated gp60 prepared by method 2.

Lane 4: TFMS treated gp60 prepared by method 2.

The single bands in lanes 2, 3 and 4 has an apparent molecular weights of 60 kD, 52 kD and 48 kD.

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Figure 3.21:

Immuno-electrophoresis of normal serum (well 1),
gp60 purified by method 2 (well 2) and
neuraminidase treated gp60 (well 3). Antiserum
to whole human serum is in troughs a and c, and
anti-gp60 is in trough b. Anode to the left.

a



b



c



d



Figure 3.22:

Determination of the true molecular weight of gp60. The molecular weight of purified gp60 was determined by SDS-PAGE analysis using a series of different gel concentrations. The apparent molecular weight was plotted against the corresponding gel concentration. The true molecular ^{weight} of gp60 at 0% gel concentration was shown to be 43 kD.

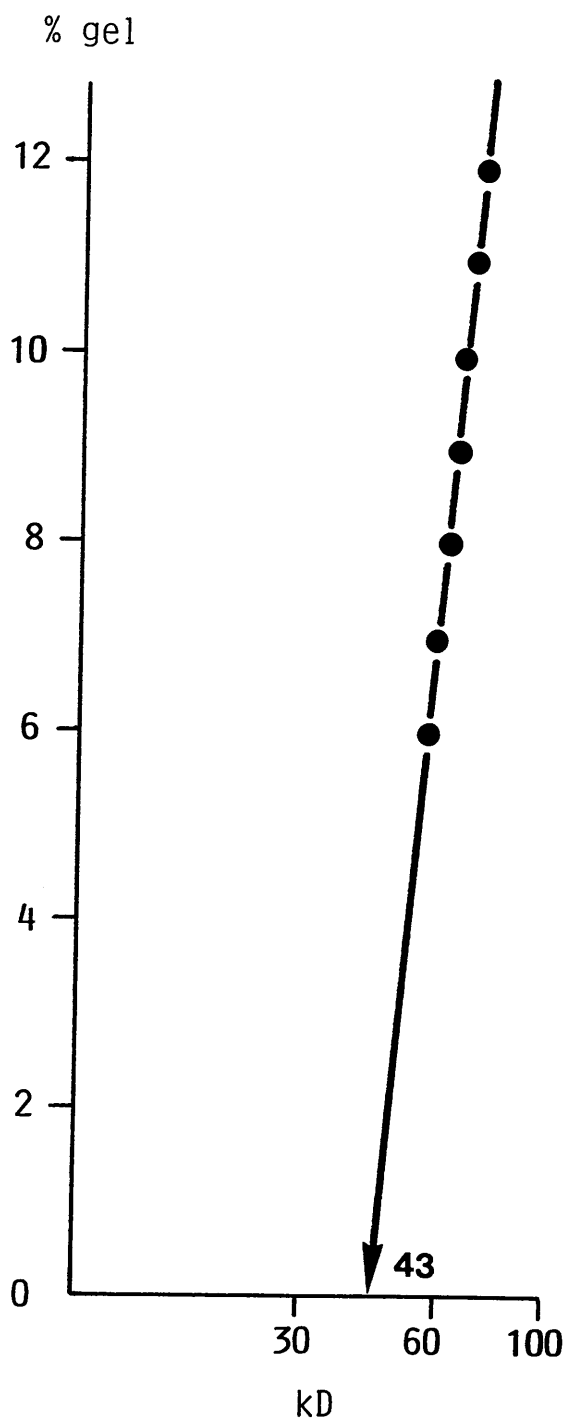


Figure 3.23:

Autoradiograph of SDS-PAGE of limited
125
proteolysis of [I]-gp60 by trypsin run under
reducing conditions. The lanes were loaded as
follows:

Lane C: control

Lanes 1 and 4 trypsin/gp60 (1/10 ratio)

Lanes 2 and 5 trypsin/gp60 (1/100 ratio)

Lanes 3 and 6 trypsin/gp60 (1/1000 ratio)

Lanes 1, 2 and 3 were digested for 1 hour at
°
37 C

Lanes 4, 5 and 6 were digested for 24 hrs at
°
37 C.

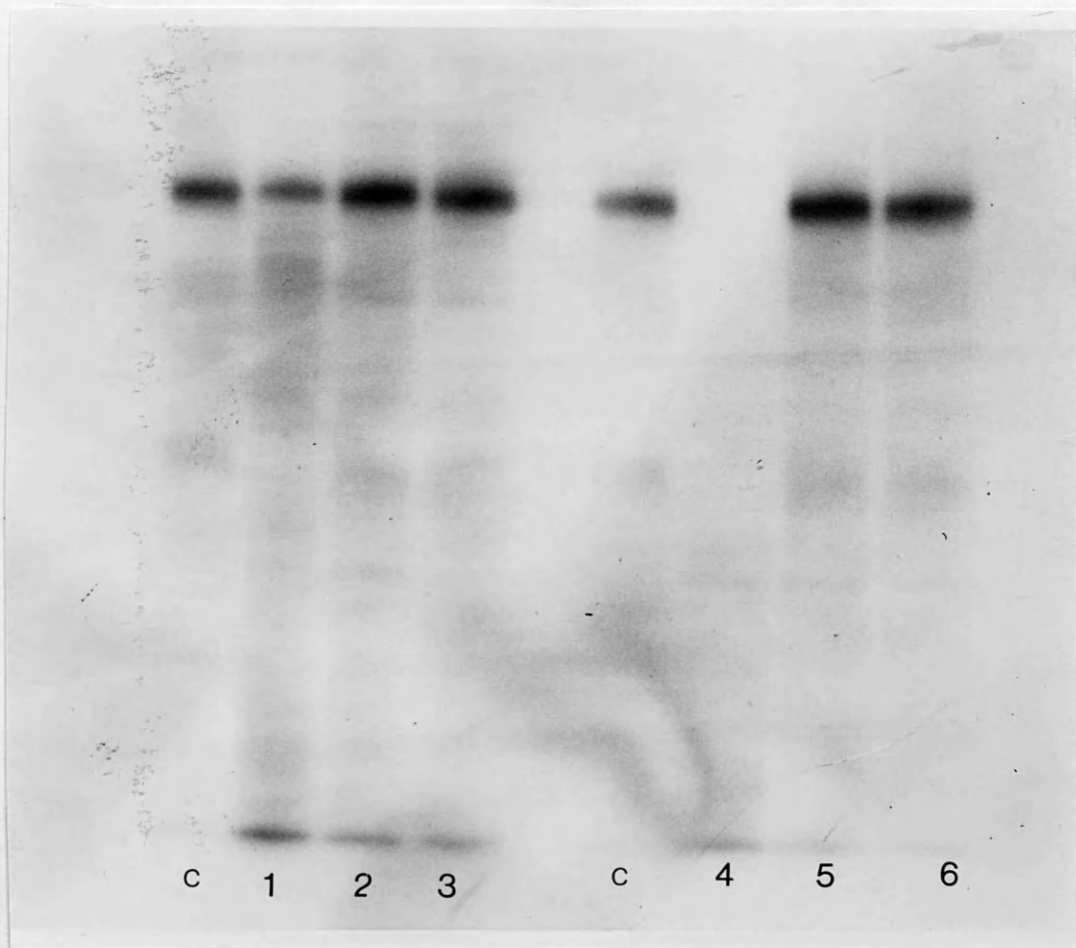


Figure 3.24:

Chymotrypsin limited proteolysis of ¹²⁵[I]-
gp60. symbols and conditions are the same as
figure 3.23.

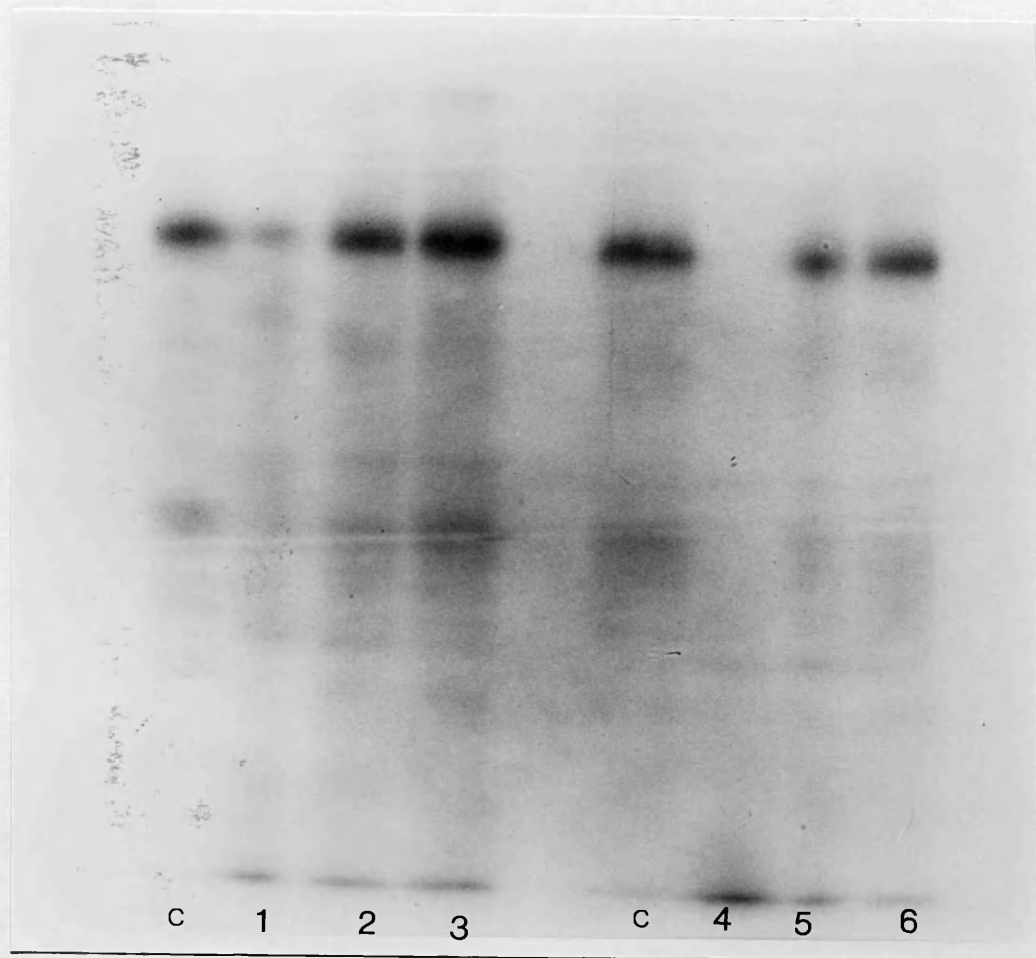


Figure 3.25:

Autoradiograph of SDS-PAGE of limited proteolysis
of ¹²⁵I-gp60 by V8 protease run under
reducing conditions. The lanes were loaded as
follows:

Lane C: control ¹²⁵I-gp60
Lanes 1 and 4: V8 protease/gp60 (1/10 ratio)
Lanes 2 and 5: V8 protease/gp60 (1/100 ratio)
Lanes 3 and 6: V8 protease/gp60 (1/1000 ratio).

Lanes 1, 2 and 3 were digested for 1 hour at
37 C.

Lanes 4, 5 and 6 were digested for 24 hrs at
37 C.

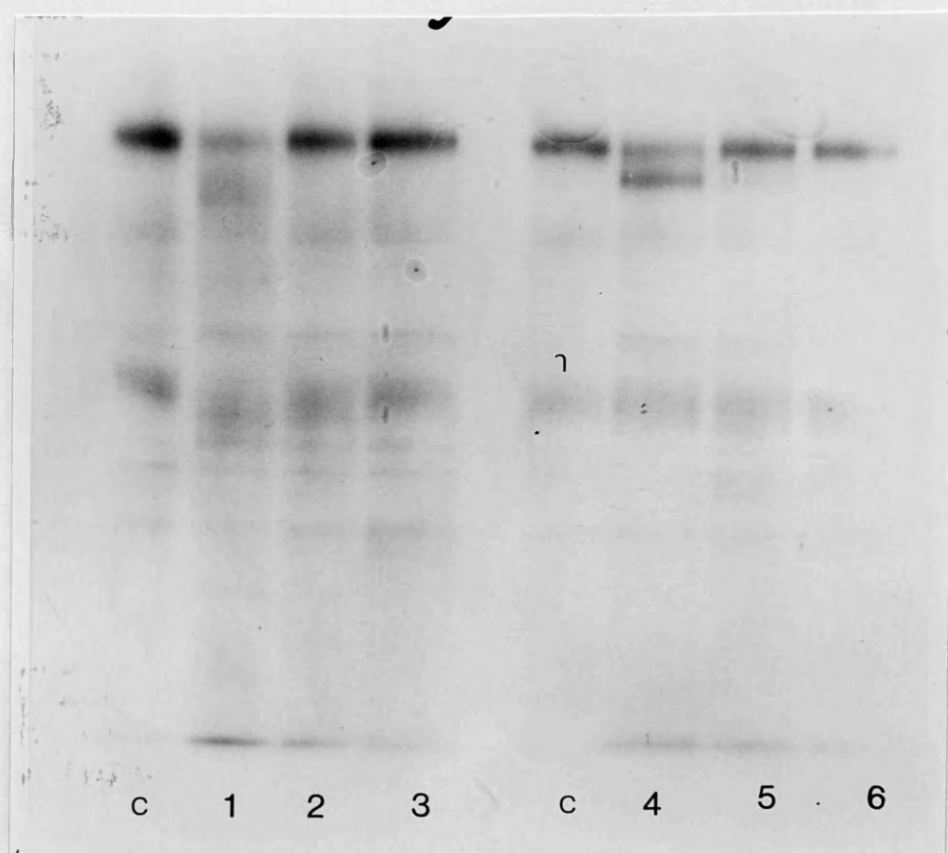


Figure 3.26:

Autoradiograph of SDS-PAGE of limited proteolysis
of ¹²⁵I]-gp60 by elastase run under reducing
conditions. The lanes were loaded as in figure
3.25.

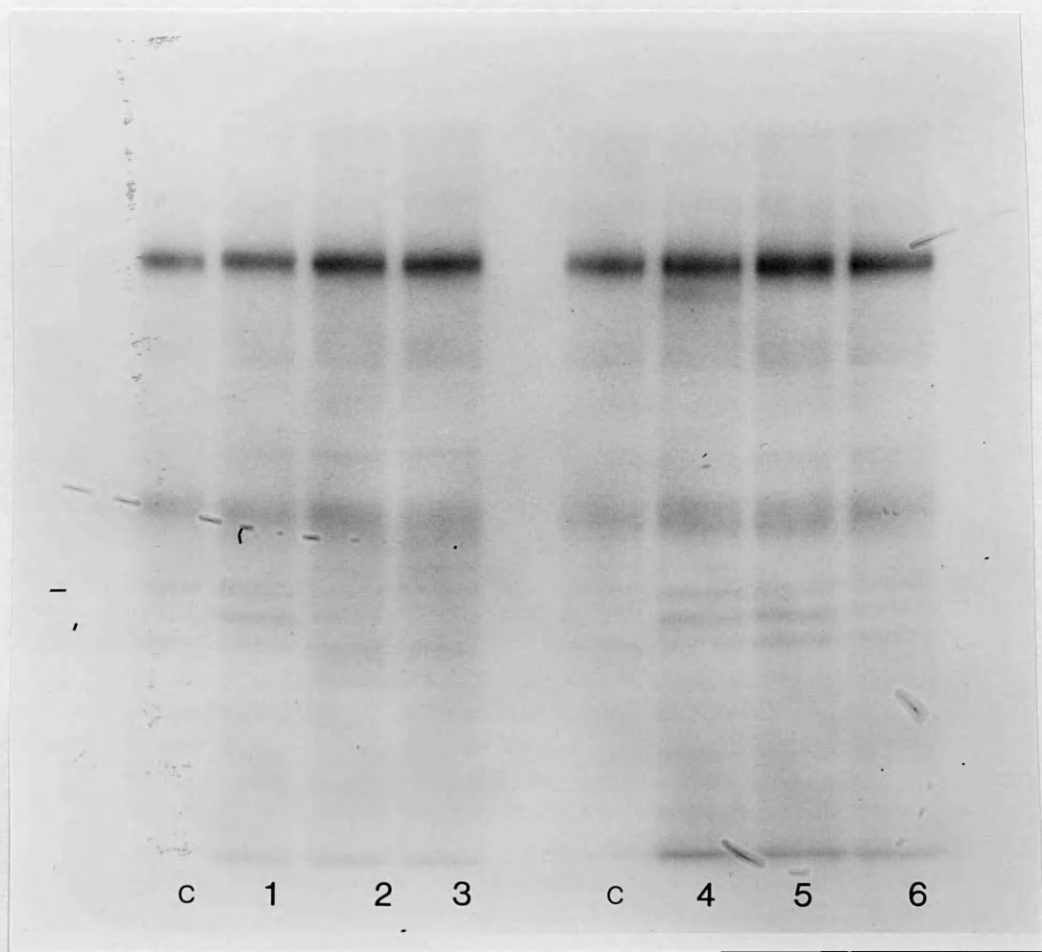


Figure 3.27:

Autoradiograph of SDS-PAGE of limited proteolysis
of reduced and alkylated ¹²⁵I-gp60 by trypsin
run under reducing conditions. The lanes were loaded
as follows:

Lane C: Reduced and alkylated ¹²⁵I-gp60.

Lanes 1 and 6: trypsin/gp60 (1/10 ratio)

Lanes 2 and 7: trypsin/gp60 (1/100 ratio)

Lanes 3 and 8: trypsin/gp60 (1/1000 ratio)

Lanes 4 and 9: trypsin/gp60 (1/10000 ratio)

Lanes 5 and 10: trypsin/gp60 (1/100 000 ratio)

Lanes 1, 2, 3, 4 and 5 were digested for 1 hr
at 37 °C.

Lanes 6, 7, 8, 9 and 10 were digested for 24 hrs
at 37 °C.

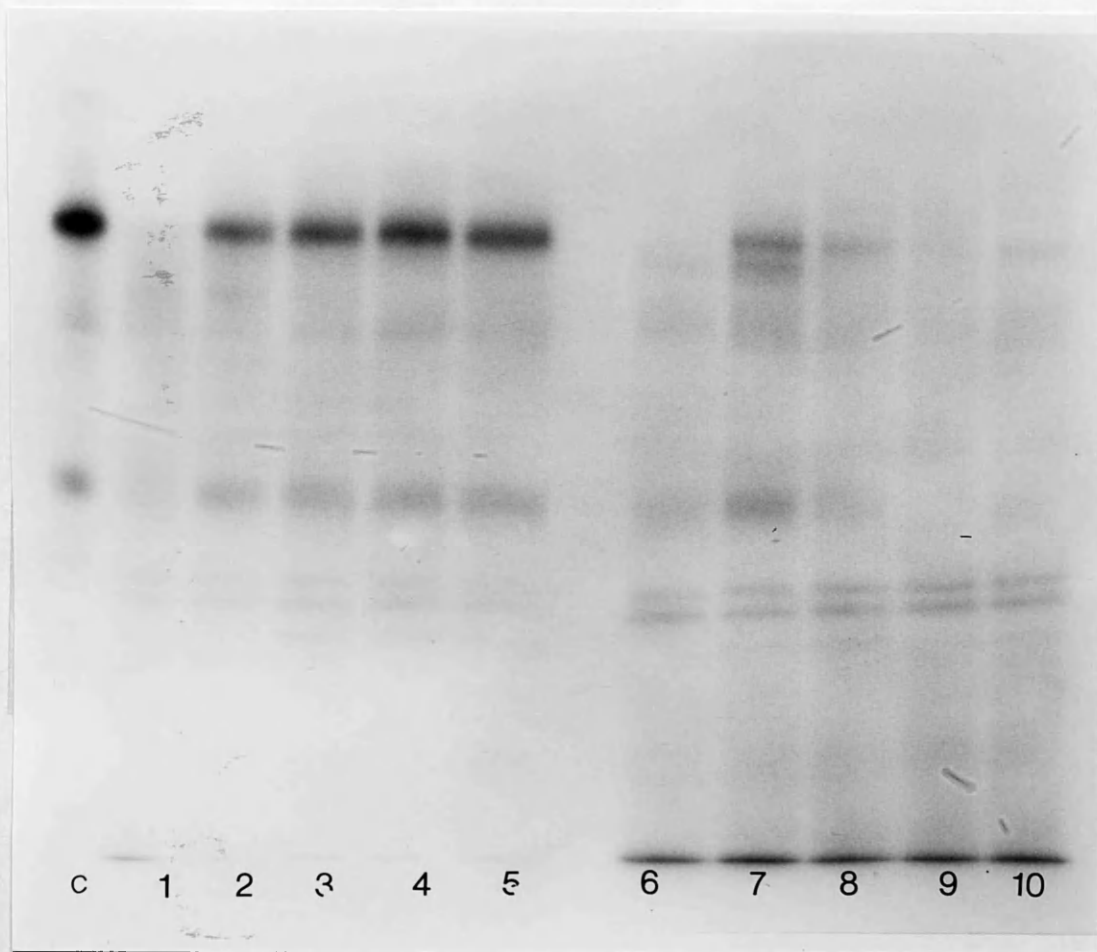


Figure 3.28:

Autoradiograph of SDS-PAGE of limited proteolysis
of reduced and alkylated ¹²⁵I]-gp60 with
chymotrypsin. The lanes were loaded as in
figure 3.21.

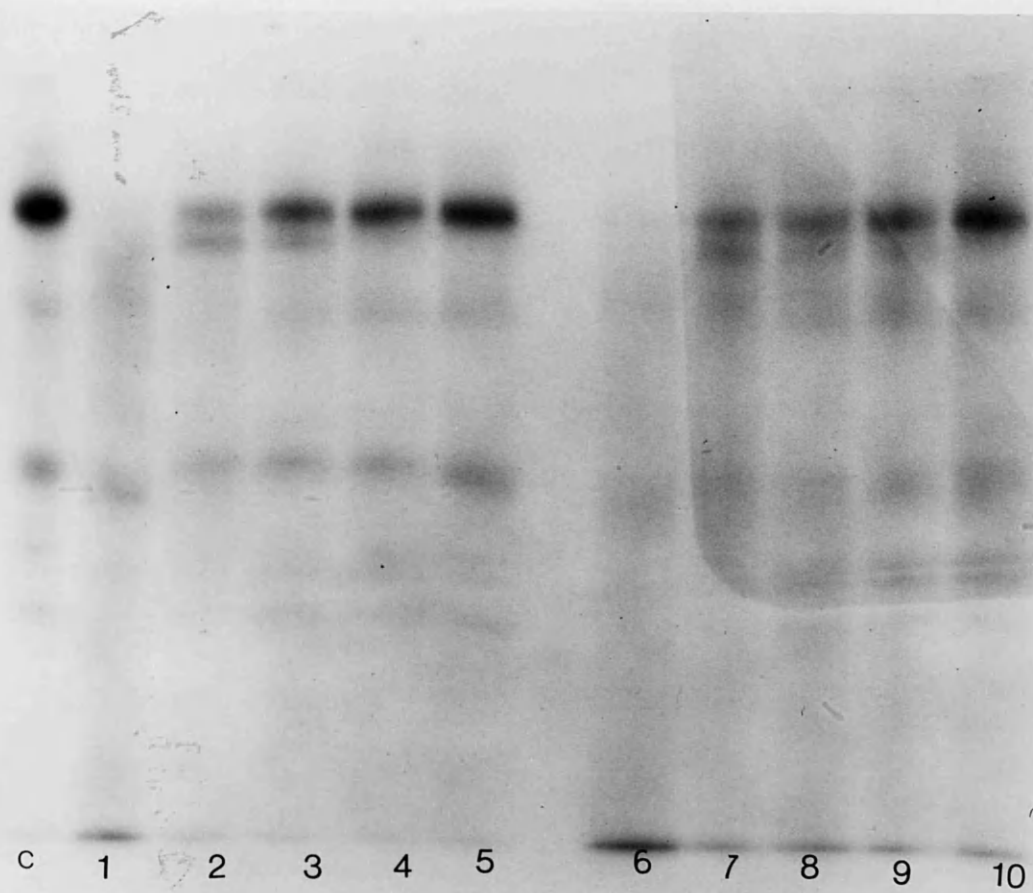


Figure 3.29:

Autoradiograph of SDS-PAGE of limited proteolysis
of reduced and alkylated ¹²⁵[II]-gp60 with
V8 protease. The lanes were loaded as described
in figure 3.27.

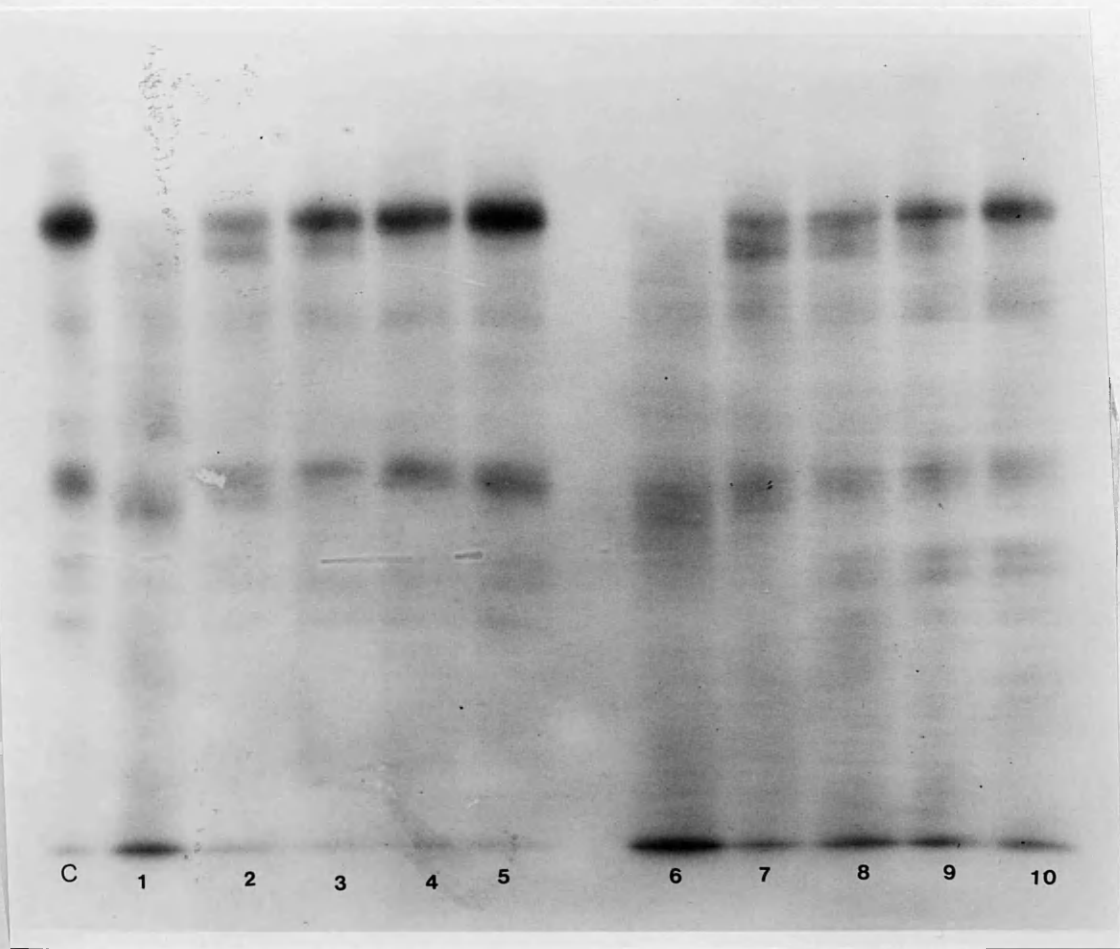


Figure 3.30:

Autoradiograph of SDS-PAGE of limited proteolysis
of reduced and alkylated ¹²⁵I-gp60 by
elastase. The lanes were loaded as in figure 3.27.

3.2.8 ANTISERUM PRODUCTION

On double diffusion against normal human serum, anti-gp60 produced a single precipitin line which gave a reaction of complete identity with all three purified gp60 preparations (Fig. 3.31).

On immunoelectrophoresis of normal human serum, anti-gp60 produced a single precipitin arc which overlapped the β 2 and γ regions (Fig. 3.16).

The specificity of the antiserum was further confirmed by immunoblotting analysis of normal human serum and purified gp60 (prepared by method 1) on 10% SDS-PAGE, in which the IgG fraction of the antiserum detected a single band with a molecular weight of 60 kD (Fig. 3.32). This band was not seen when normal rabbit IgG was used. Furthermore, the antiserum did not cross-react with 26 antisera to different plasma proteins (Table 3.4) on double diffusion in agarose gels. The antiserum was also analysed by Dr Chester Alper, (Centre for Blood Research, Boston, Massachusetts, USA). The antiserum was shown to react monospecifically with normal human serum, but the identity of the protein which was detected by the antiserum could not be identified. A list of the 57 antisera to different human plasma protein antisera which did not cross-react with the anti-gp60 in Dr Alper's laboratory are shown in Table 3.5.

3.2.9 Gp60 ELISA

The IgG fraction of the anti-gp60 antiserum was used for the development of an ELISA (3.1.26). The assay was

Figure 3.31:

Double-diffusion of gp60 preparations in agarose gel. Gp60 purified by method 1 (well 1). gp60 purified by method 2 (well 2), gp60 purified by method 3 (well 3) and normal human serum (well 4). Antiserum to gp60 (purified by method 1) is in the central well (5).

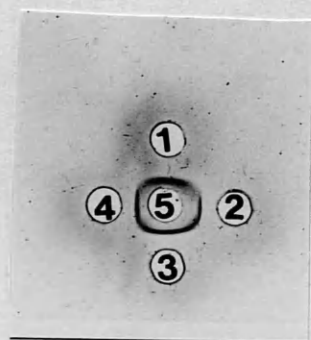


Figure 3.32:

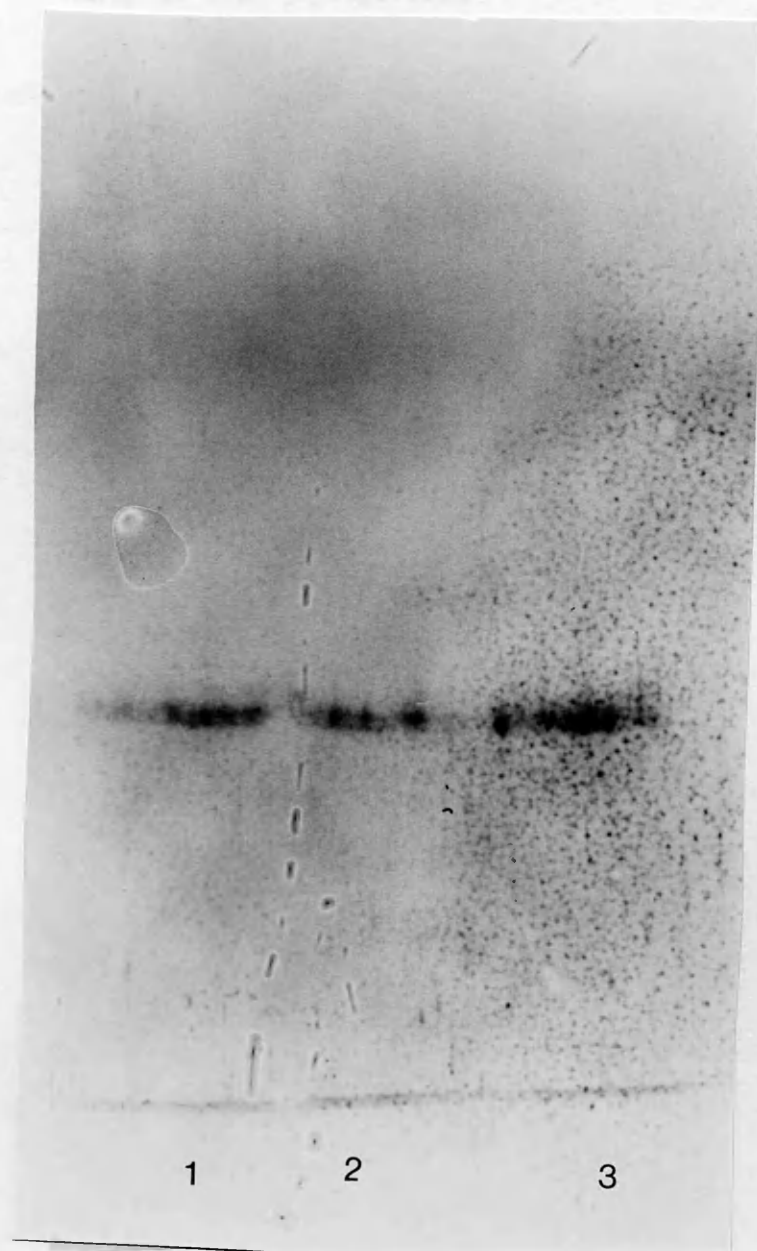
Anti-gp60 immunoblot of SDS-PAGE of normal human serum (lane 1), inhibitory peak from IgG-Sepharose column 3.1.4 (lane 2), gp60 purified by method 1 (lane 3). Lanes 1 - 3 run under reducing conditions.

TABLE 1

Summary of results of the analysis of the

samples of the

samples of the



Anti- β -globulin

Anti- γ -globulin

TABLE 3.4

Antisera which did not react with gp60

Anti-Prealbumin

Anti- α 1 acid glycoprotein

Anti-IgG

Anti-IgA

Anti-IgM

Anti-Transferrin

Anti-Albumin

Anti-Clq

Anti-Cl_s

Anti-C4

Anti-C2

Anti-C3

Anti-C5

Anti-C6

Anti-C7

Anti-C8

Anti-C9

Anti-Factor B

Anti-Factor H

Anti-Factor I

Anti-Cl-Inhibitor

Anti-C4 Binding Protein

Anti-S-protein

Anti- β ₂-glycoprotein 1

TABLE 3.5

List of antisera which did not cross-react with anti-gp60
(tested in Dr. Alper's laboratory)

Anti- B lipoprotein	
Anti-P Component of Amyloid	Anti-C-reactive protein
Anti-Thyroxine binding globulin	Anti-Ceruloplasmin
Anti-Prothombin	Anti-free lambda chains
Anti-Haptoglobin	Anti-free Kappa chains
Anti-C1 inhibitor	Anti-IgE
Anti-Gc globulin	Anti-factor B
Anti-Hemopexin	Anti-C5
Anti-IgD	Anti-Clq
Anti-Prealbumin	Anti-Clr
Anti-Transferrin	Anti-Cls
Anti-C4BP	
Anti-C8 α/γ	Anti-Factor VIII-related antigen
Anti-C3	Anti-lactoferrin
Anti-Fab k + λ light chains	Anti-Plasminogen
Anti-IgG	Anti-lysozyme
Anti-IgA	Anti-Pregnancy associated antigen
Anti-IgM	Anti-Human placental antigen
Anti-Properrdin	Anti-secretory IgA
Anti-C4	Anti-Fibrinogen
Anti-Albumin	Anti- β_2 Microglobulin
Anti-Orosomucoid	Anti- α -fetoprotein
Anti- α_1 -antitrypsin	Anti-IgG Heavy chain
Anti- α_2 -Hs glycoprotein	Anti-IgA "
Anti- β_2 -glycoprotein 1	Anti-IgM "
Anti-Factor H	Anti-IgD "

TABLE 3.5 (continued)

Anti-Gammaglobulins

Anti-IgE Heavy chain

Anti-Thrombin III

Anti-tissue A Component

Anti- α 1-antichymotrypsin

Anti- α 2-macroglobulin

Anti-Ferritin

reproducible and sufficiently sensitive to detect as little as 2.3 ng/ml gp60 without any interference from IgM-RF. The standard curve was linear up to 600 ng/ml (Fig 3.33). The intra-assay variation was determined by performing triplicate assays on each of six normal and six rheumatoid arthritis sera and was shown to be + 2.4% of the mean. The interassay variation was measured by repeating the assays on the same sera on three consecutive days and was shown to be + 5.6% of the mean (Table 3.6).

3.2.10 INHIBITION OF PIP

The addition of increasing quantities of gp60 (prepared by method one) to normal serum produced a dose-dependent inhibition of PIP, which was maximal at a concentration of 100 ug/ml (Fig. 3.34). Gp60 also inhibited the ability of normal serum to solubilise preformed IC in a dose-dependent fashion (Fig. 3.34). The inclusion of Fab fragments of anti-gp60 in the reaction mixture abrogated the inhibitory effect of gp60 on PIP, while Fab fragments of normal rabbit IgG had no effect. Gp60, purified without the IgG-Sepharose affinity chromatography (method 3), was four-times as effective as those gp60 preparations prepared by IgG affinity chromatography (methods 1 and 2) in inhibiting PIP and solubilization (Fig.3.35). All the three preparations of gp60 were much more effective at inhibiting PIP than solubilization.

Gp60 was more effective at inhibiting PIP and solubilization of IC formed at four-times antibody-excess and at equivalence than those formed at four-times antigen-

Figure 3.33:

Gp60 ELISA standard curve.

$A_{492\text{nm}}$

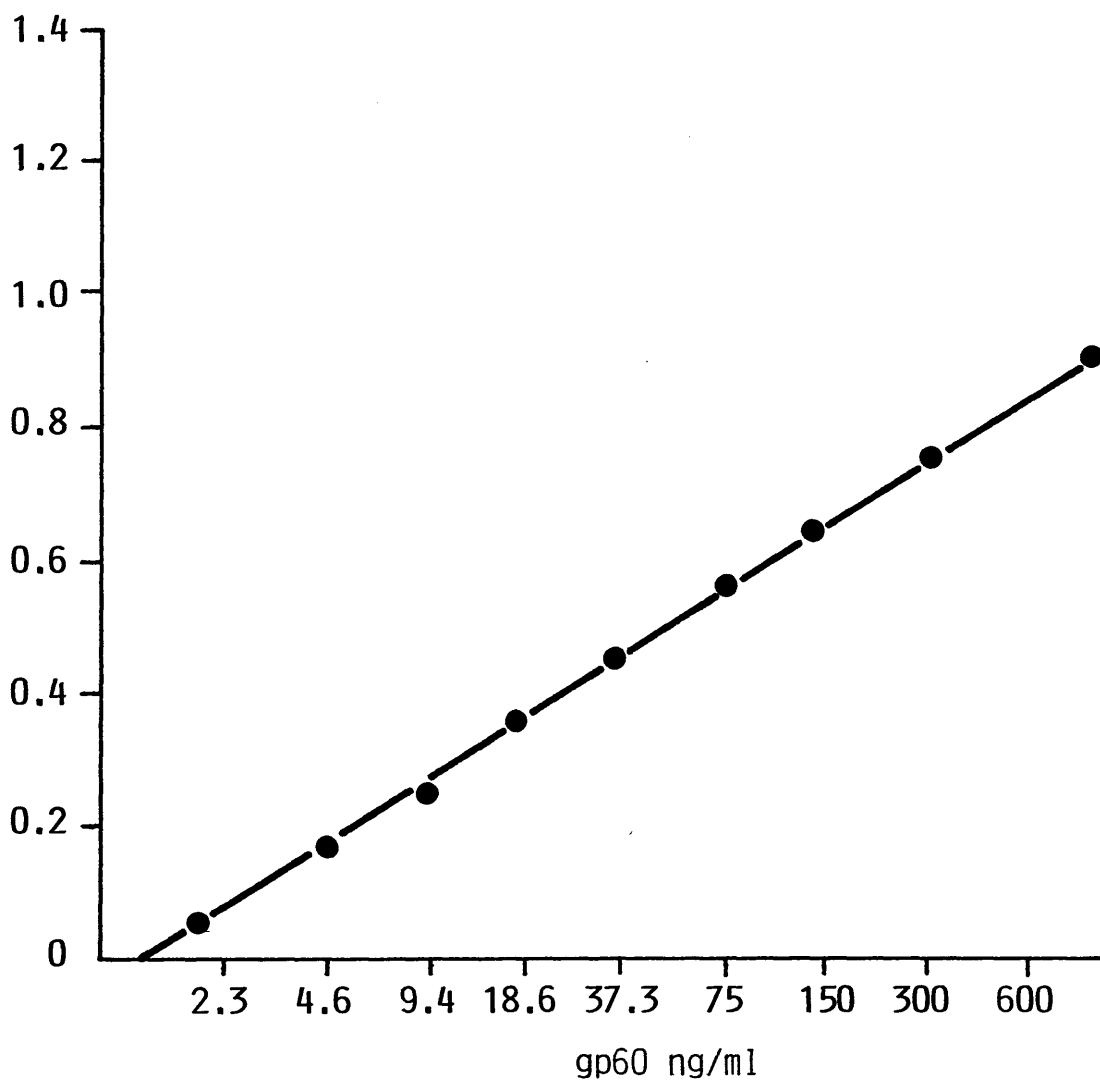


TABLE 3.6
Summary of inter- and intra-assay variations for
the gp60 ELISA

	Inter-Assay			Intra-Assay		
	Mean Conc. µg/ml	S.D.*	CV%**	Mean Conc. µg/ml	S.D.	CV%
Normal	30	2.4	0.7	32	2.8	1.6
Rheumatoid	79	5.9	1.2	84	6.1	2.4
Arthritis						

* SD = standard deviation

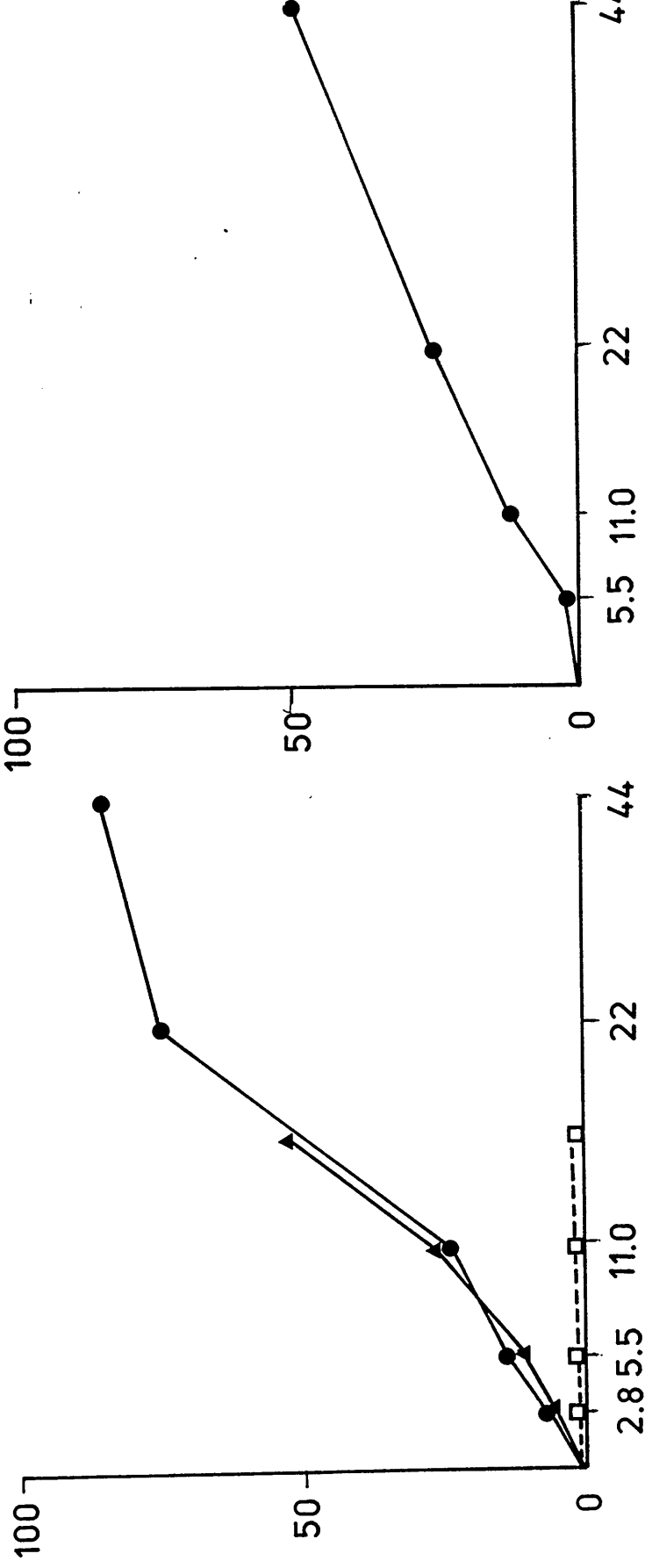
** CV% = % co-efficient of variation

Figure 3.34:

Effect of gp60 (prepared by method 1) on PIP (a) and solubilization (b). Maximum inhibition of PIP was achieved at a concentration of 100 ug/ml (●—●). Fab fragments of anti-gp60 (1 mg/ml) abolished the inhibitory effect of gp60 (□----□), while Fab fragments of normal rabbit IgG had no effect (▲—▲).

a

% INHIBITION of PIP



b

% INHIBITION of Solubilisation

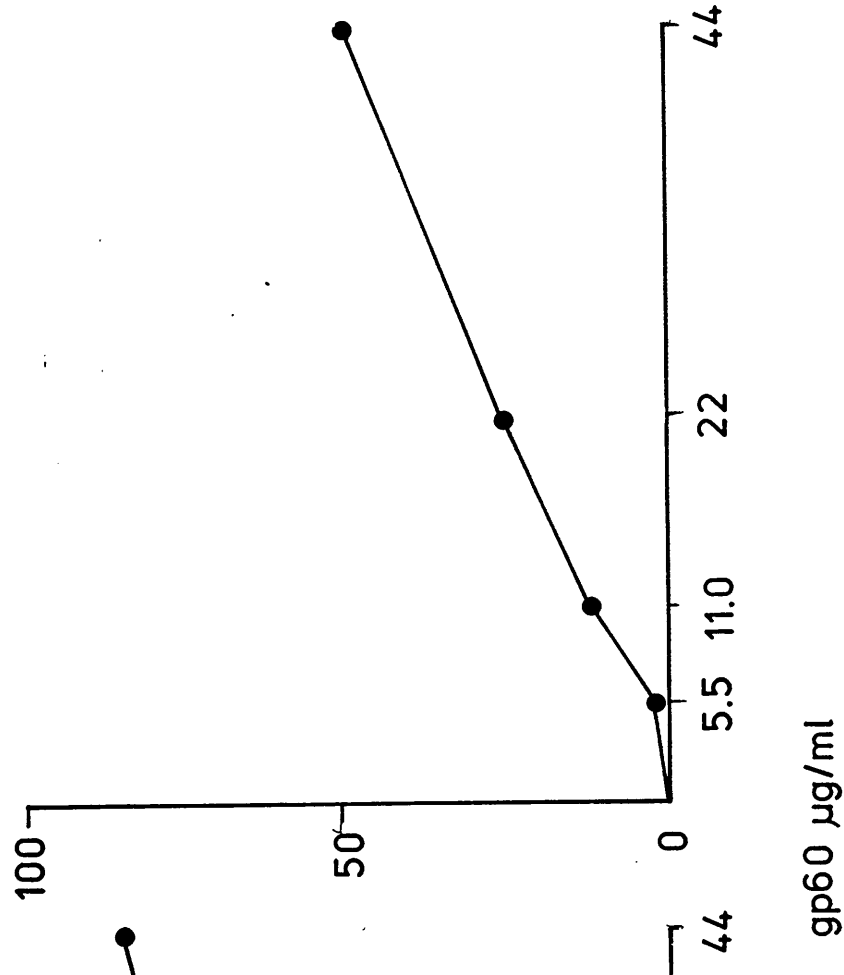


Figure 3.35:

Effect of different gp60 preparations on
PIP (a) and solubilization (b).

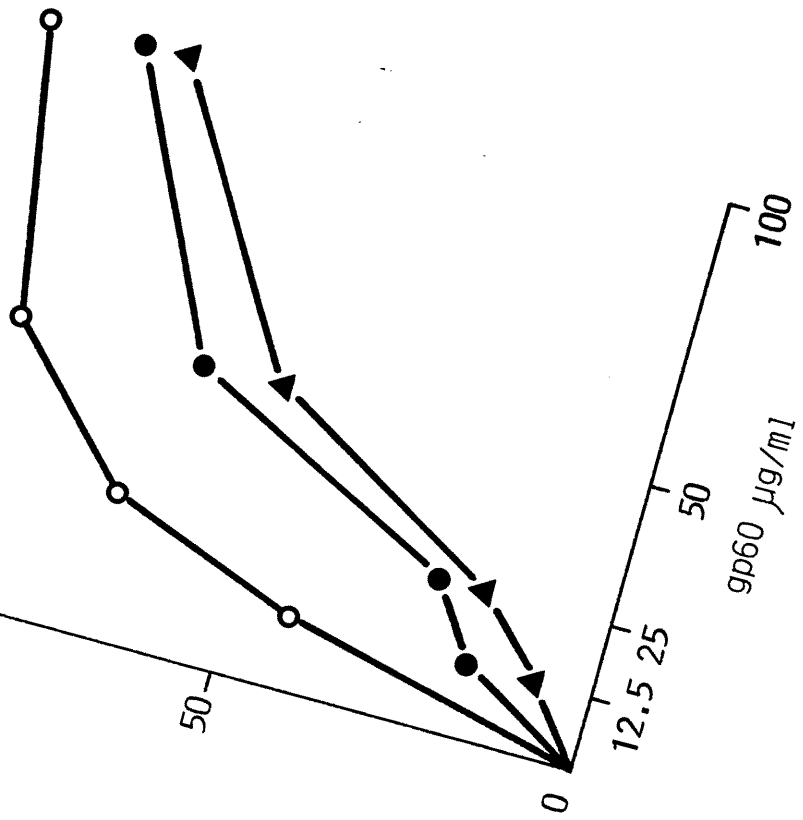
Gp60 prepared by method 1 (▲ — ▲)

Gp60 prepared by method 2 (● — ●)

Gp60 prepared by method 3 (○ — ○)

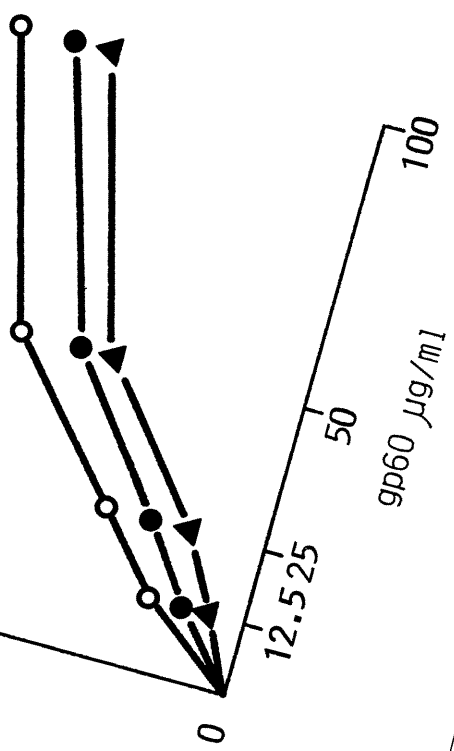
a

% inhibition of PIP



b

% inhibition of solubilisation



excess (Fig.3.36).

Kinetic studies of the effect of gp60 (prepared by method 1) on PIP and solubilisation indicated that for maximum inhibition to occur gp60 had to be present at the time of IC formation (Fig. 3.36). The percentage inhibition of PIP and solubilization decreased as the time between IC formation and the addition of gp60 to the serum increased. In the inhibition of PIP system, gp60 did not cause the reprecipitation of IC which were already soluble.

3.2.1| EFFECT OF PROTEASE INHIBITORS ON GP60 ACTIVITY

The ability of gp60 to inhibit PIP and solubilisation was not affected by it's incubation with SBTI (1 mg/ml) or PMSF (1mM) for 30 minutes at 37 ° C prior to it's use in PIP and solubilisation assays (Table 3.7).

3.2.12 EFFECT OF gp60 ON COMPLEMENT

The addition of purified gp60 to normal serum did not reduce the haemolytic activities of C4 and C2 (5.1.10; 5.1.11) during an incubation period of 30 minutes at 37 ° C (Table 3.8).

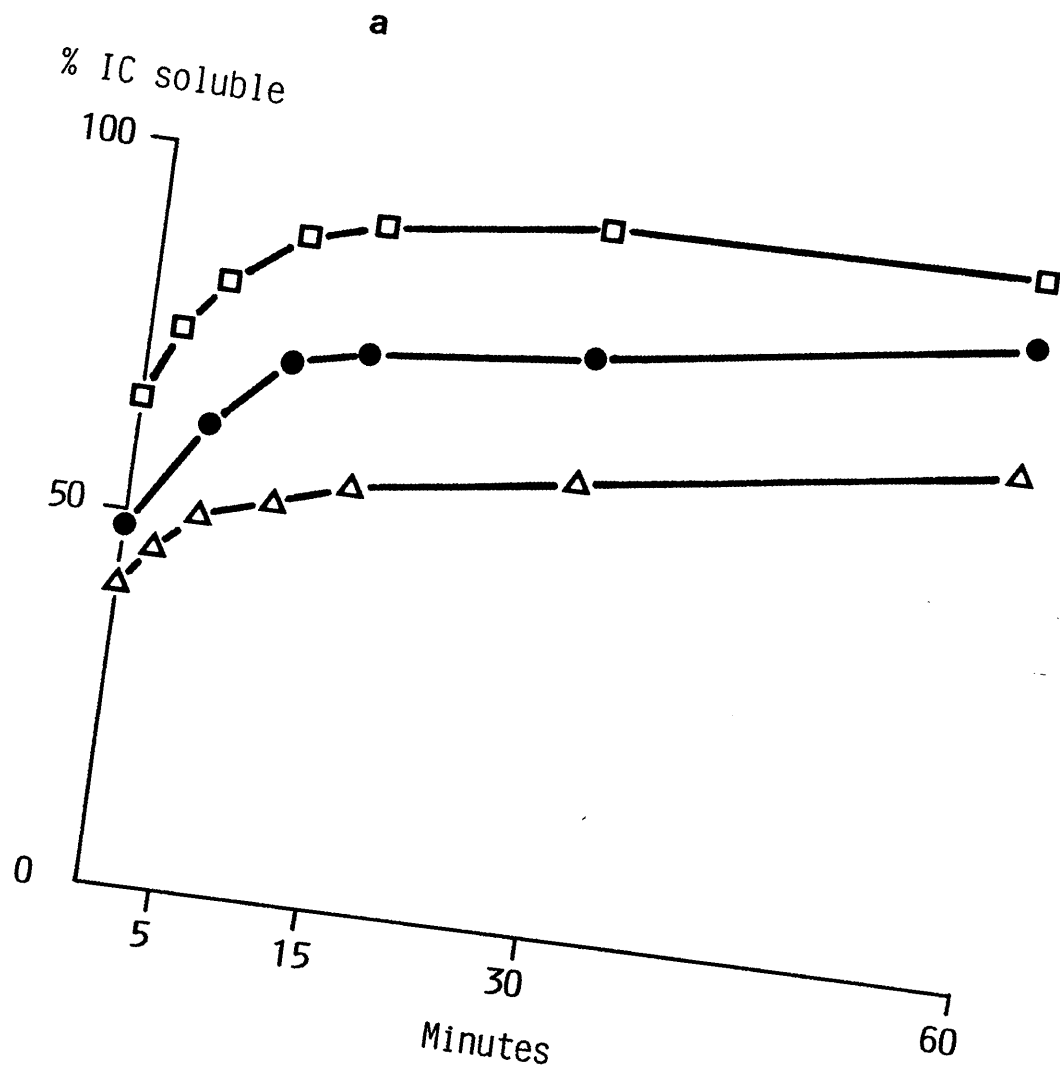
Figure 3.36:

Kinetics of gp60 action on (a)PIP and (b) solubilization.

(a) Gp60 (100 ug/ml), prepared by method 1, was added to a PIP assay at the time of formation of IC and at timed intervals afterwards. The % soluble was measured after an incubation period of 60 minutes.

(b) The same concentration of the same gp60 preparation was added to a solubilization assay at time 0 (the time of addition of the serum to the immune precipitate) or at timed intervals afterwards. The % IC soluble after 60 minutes at 37 °C was measured. IC were formed at equivalence (●—●) or at four-times antibody excess (□—□), or at four-times antigen excess (Δ—Δ).

Kinetics of action of 100 ug/ml gp60 (prepared by method 1) on PIP and solubilization of IC formed at four-times antibody excess (□—□), equivalence (●—●) and four-times antigen excess (Δ—Δ). Gp60 was added at time 0 of IC formation and 50 ul aliquotes were removed at the shown time intervals.



b

% IC soluble

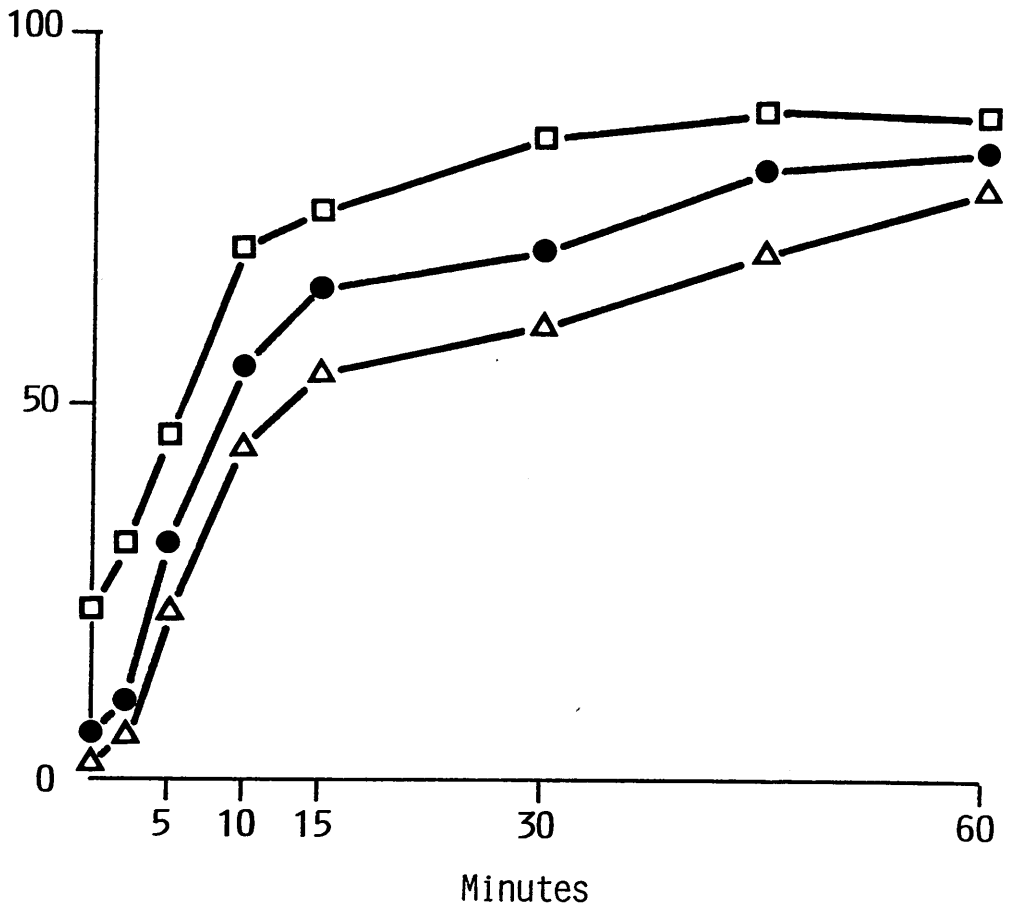


TABLE 3.7

Effect of SBTI and PMSF treated gp60 on PIP and solubilisation

% Inhibition of PIP		
gp60	+ SBTI	+ PMSF
76	74	75
52	54	51
18	18	20
9	8	8

TABLE 3.8

Effect of gp60 on normal serum C4 and C2 levels

gp60 added		C4 units/ml	C2 units/ml
100	µg/ml	22.400	2.390
50	µg/ml	24.780	2.660
25	µg/ml	25.100	2.120
12.5	µg/ml	23.560	2.650
Control		23.540	2.500

Gp60 was added to serum at the final concentrations shown prior to incubation at 37 °C for 30 minutes, after which levels of C4 and C2 were measured by haemolytic assays.

DISCUSSION

PURIFICATION OF gp60

Prior to the successful purification of gp60 from normal human serum, a number of attempts had been made to purify PIP-inhibitory material from rheumatoid arthritis serum. These preparations consisted mainly of IgM rheumatoid factor (IgM-RF) (Mitchell et al., 1984), although on SDS-PAGE analysis these preparations also contained a 60 kD band in addition to the 70 kD heavy chains and 25 kD light chains of IgM (Mitchell et al., unpublished observations; Levine R.P personal communication). However, in addition to the report from Mitchell et al. (1984), two further papers (Balastrieri et al.; O'Sullivan et al., 1988) reported that IgM-RF inhibited PIP. Neither of these reports contained data on SDS-PAGE analysis of the IgM-RF preparations.

The observation from this laboratory that serum levels of IgM-RF did not correlate with levels of PIP-inhibitory activity (Webb et al, 1986) suggested that IgM-RF could at the most be responsible for only part of the PIP-inhibitory activity, and also suggested that a second factor (possibly the 60 kD protein seen on SDS-PAGE) played a role in the ability of rheumatoid arthritis sera to inhibit PIP. It was then decided to purify this factor from normal serum in order to avoid any problems with IgM-RF contamination. It was assumed that the low levels of PIP-inhibitory activity in normal sera were produced by the same factor as was present in R.A sera, but the serum

concentrations of the factor were much lower in normal individuals.

Using three different protocols, glycoprotein 60 (gp60) was purified on the basis of it's ability to inhibit PIP. The original protocol which involved sequential affinity chromatography on IgG-Sepharose, protein A-Sepharose and Con.A-Sepharose, resulted in an average yield of gp60 in five separate preparations of 49% (range 39%-52%), and the degree of purification was 1518-fold.

During subsequent studies on the characterisation of gp60 it was noted that the apparent molecular weight of gp60 on sucrose density gradient centrifugation and gel filtration chromatography was much smaller than that of the major portion of gp60 in whole serum (Figs 3.18; 3.19). It was considered that proteolysis of gp60 during the purification procedure could be responsible for this discrepancy.

Therefore, in subsequent purification procedures protease inhibitors were added to all buffers, and all protein pools were treated with PMSF. As a result of this modification high molecular weight gp60 was obtained using the second purification procedure, which differed from the first procedure not only in the use of protease inhibitors but also in requiring a gel filtration chromatography step to remove low molecular weight contaminants and a final chromatography on heparin-Sepharose to remove traces of IgM, C4BP and fibronectin which bind to IgG-Sepharose and exhibit similar behaviour on anion exchange and gel filtration chromatography. The tendency of gp60 to bind to Sephacryl S-300 under isotonic conditions was overcome by using 1 M NaCl in the elution buffer. In three

preparations, the average yield of gp60 was 51% (range 42% - 57%), and the degree of purification was 1956-fold.

The third purification procedure was employed in order to have preparations of gp60 which had never been in contact with IgG-Sepharose. It was intended to study the binding of gp60 to complexed IgG, and the harsh conditions required for the elution of gp60 from IgG-Sepharose might have denatured the molecule irreversibly. Furthermore, it is conceivable that some gp60 might remain bound irreversibly to the affinity matrix. The final procedure involved anion exchange chromatography on DEAE-Sephacel, cation exchange chromatography on Biorex-70, gel filtration chromatography on Sephacryl-S300 followed by heparin-Sepharose chromatography. As in the second purification method, heparin-Sepharose chromatography was required to remove traces of IgM, C4BP and fibronectin. The average yield of gp60 from three preparations was 42% (range 38% - 46%), and the degree of purification was 3360-fold. The criteria of purity were a single band on SDS-PAGE (Fig. 3.14), a single precipitin line on double diffusion in agarose gel and immunoelectrophoresis against antiserum to whole human serum. (Fig. 3.17). Furthermore, rabbits immunised with purified gp60 produced a monospecific antiserum.

MOLECULAR WEIGHT OF gp60

On SDS-PAGE analysis gp60 appeared as a single band of 60 kD under both reducing and non-reducing conditions (Fig. 3.14).

Sucrose density gradient studies of whole human serum showed that the majority of gp60 sedimented with a coefficient of 19 Svedberg units (19 S), with a minor proportion sedimenting with a coefficient of 2.9 S. (Fig. 3.18). Likewise on gel filtration chromatography most of the gp60 in serum was eluted in a volume which corresponded to a molecular weight of 1000 kD with a minor proportion having an apparent molecular weight of 58 kD (Fig. 3.19).

Gp60 which had been purified by the first procedure had the same sedimentation and gel filtration characteristics as the minor serum component, hence the possibility that gp60 had been proteolytically digested during purification was considered. The incorporation of protease inhibitors into the subsequent purification procedures resulted in gp60 preparation which had sedimentation and gel filtration characteristics of the larger molecular weight fraction of serum gp60, which appeared to confirm this possibility. However, as gp60 purified by all three procedures appeared as a single band on SDS-PAGE run under reducing and non-reducing conditions, it is probable that the high molecular weight form of gp60 represents a polymer of the smaller molecular weight material, which is probably gp60 monomer.

The larger and smaller molecular weight forms of gp60 gave a reaction of complete identity on double diffusion in agarose gel using antisera raised against both the purified high molecular weight and low molecular weight preparations. Thus, there do not appear to be any major antigenic differences between the high and low molecular weight forms of gp60.

Using a molecular weight 1000kD for the polymer and 60 kD for the polymer and 60 kD for the monomer it would appear that the polymeric form of gp60 is made up of 16 monomeric subunits. However, it is difficult to explain why gp60 dissociated into the smaller molecular weight form when purified in the absence of protease inhibitors. The purified protein was relatively resistant to proteolysis as judged by SDS-PAGE analysis. However, it must be emphasised that serum proteases were not studied. Furthermore, a single proteolytic cleavage which resulted in dissociation of the larger form into the monomeric subunits may not have been detected by this technique. Although the hypothesis that serum gp60 comprises a polymer, which is assembled from a number of monomeric subunits, has not yet been proven, for the sake of simplicity in the remainder of the thesis, the larger molecular weight form will be called gp60 polymer and the smaller molecular weight form called gp60 monomer.

GLYCOPROTEIN NATURE OF gp60

Although gp60 is relatively resistant to proteolysis as shown by the high enzyme:substrate ratios (1:10;1:100 for trypsin chymotrypsin, elastase and V8 protease) and the long incubation periods (1-24 hours) required for digestion, these data confirm the protein nature of gp60. Interestingly, reduction and alkylation of gp60 did not increase its sensitivity to proteolytic enzymes to any great extent. The observation that the gp60 band in polyacrylamide gels stained in the PAS reaction indicated

that the molecule contained carbohydrate and hence was a glycoprotein with an apparent molecular weight of 60 kD. It was therefore called glycoprotein 60 (gp60). Gp60 was shown to be sensitive to neuraminidase by the more cathodal migration on immunoelectrophoresis of the neuraminidase digested preparation compared with untreated gp60 (Fig. 3.21). This result was confirmed by the observation that on SDS-PAGE analysis the apparent molecular weight of neuraminidase treated gp60 was reduced to 52 kD. Removal of all the carbohydrate with TFMS reduced the apparent molecular weight of gp60 to 48 kD on SDS-PAGE. This result was supported by the results of measuring the apparent molecular weight of gp60 by SDS-PAGE using a series of gels of different polyacrylamide content. This experiment gave a real molecular weight for gp60 of 43 kD.

OTHER CHARACTERISTICS OF gp60

On isoelectric focusing, gp60 in whole serum and both the polymeric and monomeric forms of purified gp60 had isoelectric points of 5.1-5.3. On immunoelectrophoresis the polymeric form of gp60 had β_2/γ globulin mobility, while the monomeric form migrated as a pre-albumin. The large molecular weight of gp60 polymer makes it difficult for the molecule to migrate through the agarose gel so the precipitin arc appears at the boundary zone between the β_2 and the γ globulin fractions.

When serum was electrophoresed in agarose gel and the gel sliced and the gp60 eluted from the slices, gp60 (measured by ELISA) was found principally in the β_2/γ globulin boundary zone, but a significant amount was also

found in the pre-albumin region (Fig. 3.37). This confirms the result of the sucrose density gradient centrifugation and the gel filtration studies which demonstrated that a proportion of serum gp60 was in the monomeric form.

The inhibitory action of gp60 on PIP was not due to serine protease activity as SBTI and PMSF treated gp60 was still able to inhibit PIP.

The protein content of purified gp60 preparations was measured by O.D₂₈₀, Folin assay and by the BCA technique which is a modified Biuret assay. The former procedures which detect aromatic amino acids gave values six times higher than those of the BCA technique, which measures peptide bonds. This discrepancy could be accounted for by a high aromatic amino acid content of gp60. Although we have not yet obtained a complete amino acid analysis, preliminary data do suggest that gp60 has an extremely high tryptophane content (Willis A et al, unpublished observations). Thus for all the data reported in this thesis, the protein content of purified gp60 preparations was based on results of the BCA procedure.

Gp60 INHIBITS PIP AND SOLUBILISATION

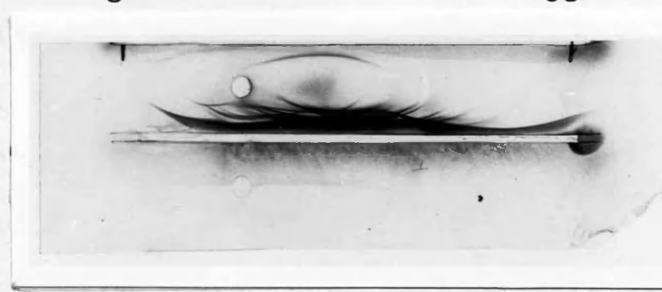
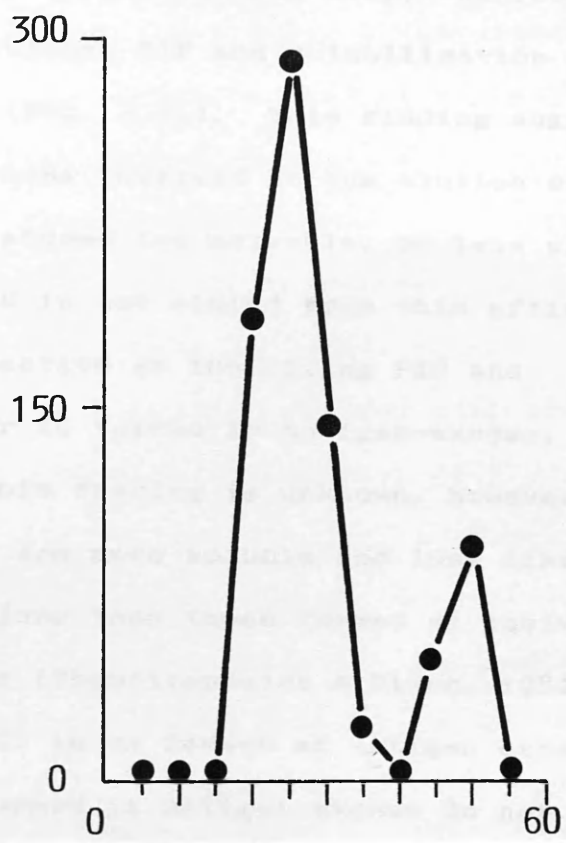
Both monomeric and polymeric forms of gp60 were able to inhibit PIP and solubilisation. These effects did not occur in the presence of Fab fragments of anti-gp60, although Fab fragments of normal rabbit IgG did not affect these activities of gp60. The monomeric form of gp60 and the polymeric form which had been purified by IgG-Sepharose affinity chromatography were almost equally effective at

Figure 3.37:

Immuno-electrophoresis of normal human serum
(well 1), anti-whole human serum is in trough a
and anti-gp60 is in trough b.

An identical part of the gel was cut into 2.0 mm
slices, which was eluted for 48 hours at 4 °C in
PBS. Gp60 concentration in the eluate was
measured by ELISA.

gp60 ng/ml



inhibiting PIP and solubilisation. In contrast, polymeric gp60 which had been purified by method 3 (i.e no IgG-Sepharose affinity chromatography step) was four-times more effective at inhibiting PIP and solubilisation as the other two preparations (Fig. 3.35). This finding suggests that either the conditions involved in the elution of gp60 from IgG-Sepharose denatured the molecule, or less probably, high affinity gp60 is not eluted from this affinity matrix. Gp60 was less effective at inhibiting PIP and solubilisation for IC formed in antigen-excess. The significance of this finding is unknown, however IC formed in antigen excess are more soluble and less likely to produce tissue injury than those formed at equivalence or in antibody excess (Theofilopoulos & Dixon, 1980). It is also unusual for IC to be formed at antigen excess in-vivo. Furthermore, IC formed in antigen-excess do not activate complement as well as those formed at equivalence or in antibody excess (Theofilopoulos & Dixon, 1979). Thus, if gp60 inhibits PIP by interfering with the ability of complement to retain in solution IC formed at equivalence or in antibody-excess, a significant pathogenetic role for gp60 could be postulated.

The dose response curves showed that gp60 was more effective at inhibiting PIP than solubilisation. As the amount of IC used in the solubilisation assays was only one quarter of that used in PIP assays, the relative resistance of solubilisation to the inhibitory action of gp60 is further emphasised. PIP is absolutely dependent upon the classical pathway (Schifferli et al., 1981; Naama

et al., 1983) and while solubilization is absolutely dependent on the alternative pathway (Czop & Nussenzweig, 1976), the process is more effective in the presence of classical pathway activation (Czop & Nussenzweig, 1976). As gp60 binds to IgG-Sepharose, and as the ability of rheumatoid arthritis sera to inhibit PIP correlated with their ability to inhibit C1q binding to IC (Niven & Whaley, 1986), and to inhibit C4 consumption by IC (Naama et al., 1984), it was likely that gp60 bound to the Fc piece of IgG and inhibited classical pathway activation. This would explain the relative sensitivity of PIP to the action of gp60.

The studies of the kinetics of the action of gp60 on PIP and solubilisation showed that for maximum effect gp60 had to be present at the time of formation of the IC. After IC had been rendered soluble by complement, gp60 did not cause IC to precipitate. Thus, gp60 acts on IC before complement activation occurs, and does not act on complement-reacted IC. Thus, these data support the earlier argument that gp60 binds to IC by the IgG antibody moiety and then interferes with complement activation.

PRODUCTION OF ANTISERUM TO gp60 AND ESTABLISHING ELISA

The rabbit antisera produced against gp60 were shown to be monospecific by double diffusion in agarose gels and immuno-electrophoresis against normal human serum (Fig. 3.16). The specificity of the first antiserum was demonstrated by the ability of Fab fragments of the IgG fraction of the antiserum to inhibit the action of gp60 on PIP and solubilisation (Fig. 3.34). All antisera

precipitated with purified gp60 on double diffusion in agarose gels and on immunoelectrophoresis.

The identity of the protein recognised by the antisera was tested in this laboratory (Table 3.4) and in the laboratory of Dr Chester Alper (Table 3.5) . The serum protein detected by anti-gp60 was not detected by monospecific antisera to 57 known plasma proteins (Table 3.5). Thus gp60 appears to be a previously undescribed plasma protein.

The gp60 antiserum was used to establish an ELISA procedure to measure gp60 levels in biological fluids. F(ab')₂ fragments were used to coat the wells of microtitre plates in order to prevent IgM-RF interfering with the assay by binding to the Fc piece of the intact antibody. The probe used to detect bound gp60 was intact IgG which had been labelled with biotin. The intact IgG was used as it was felt unnecessary to use F(ab')₂ fragments at this stage as IgM-RF would not have bound to the wells, and so would not be a source of interference. The assay was sensitive over a wide range of concentrations, the dose-response curve being linear from 2.3 ng/ml to 600 ng/ml. The assay was reproducible as shown by the low interassay and intra-assay variations (Table 3.6).

The assay was used successfully to measure gp60 in serum fractions from sucrose density gradient and gel filtration chromatography, and revealed the presence of the polymeric form of gp60 in whole serum. It was later used to measure gp60 concentrations of purified preparations used in the experiments described in chapters 4 and 5 and in

whole serum (see chapter 5).

CHAPTER FOUR

BINDING OF GP60 TO IMMUNOGLOBULINS

INTRODUCTION

The initial step in gp60 purification, affinity-chromatography on IgG-Sepharose, indicated that binding of gp60 to IgG probably played an important role in its ability to inhibit PIP. In this chapter I describe studies in which the binding of gp60 to different immunoglobulin isotypes and the localisation of the gp60 binding site on IgG was investigated. The binding affinity of gp60 for IgG in solid-phase IC was measured, and the interactions of gp60, C1q, IgM-RF, IgG-RF and Staphylococcal protein-A are reported.

METHODS

4.1.1 Gp60 PREPARATIONS

Gp60 was purified by the three procedures described in chapter three. The protein concentration of the purified preparations was determined by ELISA (3.1.26).

4.1.2 PURIFICATION OF HUMAN C1q (Tenner, Lesavre & Cooper, 1981)

Serum from one unit of fresh frozen plasma was prepared as described in 3.1.9, and 5ml of 0.2M EDTA (pH 7.5) was added to make the final concentration 5mM. The serum was then applied to a 2.6 x 20 cm Biorex-70 column equilibrated in 50 mM sodium phosphate buffer (pH 7.3) containing 2 mM EDTA and 82 mM NaCl. After washing the column with 2 l of equilibrating buffer, until the O.D₂₈₀ of the column effluent was less than 0.01, the column was eluted with a linear salt gradient composed of 300 ml starting buffer, and 300 ml starting buffer containing 0.3M NaCl. The column was screened as in 3.1.7, and fractions containing C1q were detected by radial immunodiffusion (2.7).

The C1q pool was concentrated by ammonium sulphate precipitation and then centrifuged (8000 g at 2 °C for 30 minutes). The precipitate was dissolved in 10 ml 5xVBS and dialysed overnight at 4 °C against PBS. Finally, the C1q preparation was passed over a column (1 ml) of protein A-Sepharose to remove any IgG contaminants. The purity of the C1q preparation was tested by SDS-PAGE run under reducing and non-reducing conditions on a 5 % gel

and then stored at - 70 C.

4.1.3 HUMAN IgG SUBCLASS MYELOMA PROTEINS

Human IgG1, IgG2, IgG3, and IgG4 were immunoaffinity purified from the sera of myeloma patients by the method of Bird et al, 1984. IgG1, IgG2, and IgG3 were each free from contamination with other isotypes, but IgG4 contained traces of IgG3 as assessed by haemagglutination inhibition assays.

4.1.4 PREPARATION OF Fc FRAGMENTS FROM HUMAN IgG

Human IgG was purified from normal serum and digested with pepsin as described previously (3.1.1; 3.1.23). Following dialysis against VBS the digest was passed over a column (1.0x20 cm) of protein A-Sepharose which was washed in VBS until the O.D. of the effluent was less than 0.01. The bound IgG was then eluted with 0.1 M glycine-HCl(pH 3.0) and dialysed against PBS. Five millilitres (5ml) were gel filtered on a Sephacryl-S300 column equilibrated in PBS at a flow rate of 15 ml/hr, and 2 ml fractions were collected.

The fractions were screened as in 3.1.7, and tested by SDS-PAGE run under reducing and non-reducing conditions in 10% gel.

4.1.5 PURIFICATION OF HUMAN IgM PARAPROTEIN

Two millilitres (2ml) of IgM myeloma serum were dialysed overnight at 4 C against 10 mM sodium phosphate buffer (pH 7.0). The euglobulin precipitate was recovered by centrifugation (8000 g for 15 minutes at 4 C), and

dissolved in 1 ml 5xVBS prior to dialysis against 2 l PBS. The solution was then absorbed with 100 ul of packed protein A-Sepharose for one hour at room temperature and the supernatant was collected. The purity of the preparation was tested by double diffusion in agarose gel against both anti-whole human serum and anti-IgM, and also by SDS-PAGE run under reducing and non-reducing conditions in a 10% gel.

4.1.6 PURIFICATION OF HUMAN IgA PARAPROTEIN

The euglobulin precipitate of 2 ml IgA myeloma serum was formed and collected as in 4.1.5. The precipitate was dissolved in 1 ml 5xVBS and then dialysed against 10 mM Tris-HCl (pH 8.0) prior to absorption onto DE52 (100 ul of 50% suspension in 10 mM Tris-HCl, pH 8.0). The supernatant was discarded, and after washing the matrix several times with starting buffer, the IgA was eluted by adding 1 ml of starting buffer containing 0.5 M NaCl to the ion exchange pellet. IgA purity was confirmed by double diffusion in agarose gel against anti-whole human serum and anti-IgA, and by SDS-PAGE run under reducing and non-reducing conditions in a 10 % gel.

4.1.7 PREPARATION OF IgM AND IgG RHEUMATOID FACTORS (Mitchell et al, 1984)

Rheumatoid arthritis serum (20 ml) was applied to an IgG-Sepharose column (2.5x15 cm) equilibrated in 10 mM Tris-HCl buffer (pH 8.0) containing 10 mM EDTA and 0.15 M NaCl at a flow rate of 20 ml/hr. After washing the column with several column volumes of the starting buffer, IgM-RF

and IgG-RF were eluted with 0.1 M sodium acetate buffer (pH 4.0) containing 0.5 M NaCl. The two rheumatoid factors were separated on Sephacryl-S300 column (2.6x90 cm) equilibrated in 0.1 M sodium acetate (pH 4.0) containing 0.5 M NaCl, at a flow rate of 15 ml/hr and 2 ml fractions were collected. IgM-RF was confined to the first protein peak, whereas IgG-RF was present in the second peak. The fractions containing IgM-RF were pooled, dialysed against PBS and stored at 4 °C, while those which contained IgG-RF were digested with pepsin and the F(ab')₂ fragments were purified as described in 3.1.23. The purified F(ab')₂ fragments were dialysed against PBS and stored at -20 °C.

4.1.8 PREPARATION OF IMMUNOADSORBENT-PURIFIED IgG ANTI-BSA

Twenty millilitres (20ml) of rabbit anti-BSA were applied to a BSA-Sepharose column (1.5 x 10 cm) equilibrated in PBS. After washing the column extensively with PBS until the O.D. of the effluent was less than 0.01, the bound proteins were eluted with 0.1M glycine-HCl (pH 3.0) containing 0.5 M NaCl and antibody containing fractions were neutralised with 100 ul of 1M Tris. After pooling the anti-BSA was further purified by DE52 chromatography as described in 4.1.6.

4.1.9 RADIOLABELLING OF PROTEINS

IgG anti-BSA was labelled using ¹²⁵I [I] by the chloramine-T method, while gp60, C1q, IgM-RF and IgG-RF were radiolabelled by the solid-phase Iodogen method

(2.14).

The specific radioactivities were; gp60 prepared by method 1 (3.4×10^9 cpm/ug), gp60 prepared by method 2 (9.6×10^8 cpm/ug), gp60 prepared by method 3 (1.2×10^6 cpm/ug), C1q (1.4×10^7 cpm/ug), IgM-RF (6.1×10^6 cpm/ug), F(ab') IgG-RF (1×10^7 cpm/ug), and IgG anti-BSA (4.2×10^8 cpm/ug).

4.1.10 BINDING OF PROTEINS TO MICROTITRE WELLS

Disposable U-shaped wells (Remova wells, Dynatech Labs Ltd.) were coated with 100 μ l of the appropriate protein solution (0.1-10 μ g/ml) diluted in 10 mM carbonate/bicarbonate buffer pH 9.6 during an overnight incubation at 4 °C.

4.1.11 PREPARATION OF SOLID-PHASE IMMUNE COMPLEXES (IC)

BSA-coated wells (1 μ g/ml) were washed five times with PBS-Tween (PBS containing 0.05% (v/v) Tween-20), and the unreacted sites were blocked with 0.1 % (w/v) gelatin in PBS (250 μ l/well) except for those wells to be used for C1q binding assays in which case 0.1% (w/v) casein was used to reduce the background binding. The ¹²⁵[I]-labelled or non-radiolabelled IgG-anti-BSA (0.1-0.5 μ g) in 100 μ l of PBS-Tween were added to each well. After one hour incubation at room temperature, the wells were emptied by aspiration and washed five times with PBS-Tween, prior to counting the radioactivity, or being used for the binding study. This method allowed the determination of the number of molecules of IgG bound to each well. For each concentration of IgG used, the variation in antibody binding between wells was + 4.0% of the mean. This variation was consistent throughout the series of experiments.

Extensive washing of the wells did not remove bound antibody under these conditions. In the experiments described, the number of bound IgG molecules ranged from 2.8×10^{10} to 6.4×10^{12} per well.

4.1.12 Gp60 BINDING TO IMMUNOGLOBULIN ISOTYPES, IgG SUBCLASSES AND FRAGMENTS

After the wells were coated with IgM, IgA, IgG, or its F(ab')₂, Fab or Fc fragments, IgG subclasses, C1q, IgM-RF or F(ab')₂ fragments of IgG-RF, and blocked as described in 4.1.11, 0.5 - 42 ng ¹²⁵[I]-gp60 (in 100 ul PBS-Tween) were added to each well. After incubation for 1 hour at room temperature, and washing five times with PBS-Tween, the bound radioactivity was counted. Background binding was determined using uncoated but blocked wells, which were processed in the same way as the wells to which the specific protein had been bound.

4.1.13 BINDING OF RADIOLABELLED PROTEINS TO SOLID-PHASE-IC

The binding of ¹²⁵[I]-gp60 (0.5-42 ng), ¹²⁵[I]-C1q (12-385 ng), ¹²⁵[I]-IgM-RF (6.9-120 ug) and ¹²⁵[I]-F(ab')₂ IgG-RF (6.25- 200 ng) in PBS-Tween (100 ul) to wells containing 1-8 ng IgG IC was studied. After incubation of one hour at room temperature, and washing five times with PBS-Tween, the bound radioactivity was determined. Non-specific binding was calculated using BSA-coated wells without IgG anti-BSA.

4.1.14 COMPETITION INHIBITION STUDIES

Non-radiolabelled C1q, Staphylococcus protein A, IgM.RF or F(ab')₂ IgG-RF at concentrations of 25, 50, and 100 ng/100 ul of PBS-Tween, were incubated with 100 ul of ¹²⁵[I]-gp60 (1.3-42 ng) in wells containing 7.7 ng bound IgG IC (4.1.11) for one hour at room temperature, followed by washing five times with PBS-Tween and counting the bound

radioactivity.

In another series of experiments, 4.2, 8.4, and 16.6 ng/100 ul of non-radiolabelled gp60 were incubated with ¹²⁵I-[I]-C1q (12-385 ng), ¹²⁵I-[I]-IgM-RF (6.9-120 ug), or ¹²⁵I-[I]-F(ab')₂ IgG-RF (6.25-200 ng) in wells containing the same amount of IgG-IC, and the binding studies were performed as described above.

Controls for non-specific binding using BSA-coated wells but without IgG anti-BSA were included. Control values were subtracted from experimental results.

4.1.15 NUMERICAL CALCULATIONS

The gamma counter background was subtracted from all c.p.m values. The triplicate values obtained for bound c.p.m. were averaged and the standard errors calculated. Any single value differing by more than 5% of the mean of the three values was excluded from the calculation. When two values differed by more than 5% of the mean of the triplicate measurements, the experiment was repeated. All the experiments described were repeated on at least three occasions. The specific binding values were obtained by subtracting the non-specific binding values from the total binding values over a range of input values. In all experiments the radioactivity due to non-specific binding was less than 5% of the specific binding counts. Using the specific binding values at a given input, Scatchard, double-reciprocal, and Hill plots were drawn to calculate the affinity constants and to determine the number of binding sites on the IgG-IC using linear regression analysis.

The molecular weights used for the calculations were:

gp60-60 kD., C1q-410 kD., IgM-RF-960 kD., and IgG-RF

F(ab')₂ 100 kD.

2

RESULTS

4.2.1 BINDING OF gp60 TO IMMUNOGLOBULIN ISOTYPES

There was no significant binding of ¹²⁵[I]-gp60 prepared by method 1 to wells coated with IgM or IgA (Fig. 4.1), in contrast ¹²⁵[I]-gp60 was able to bind to IgG, the amount of binding being dependent upon the amount of IgG bound to each well, and upon the amount of ¹²⁵[I]-gp60 added to each well (Fig. 4.1).

Kinetic studies of ¹²⁵[I]-gp60 binding to IgG-coated wells was rapid with 90-95% of maximum binding occurring within 10 minutes (Fig. 4.1).

4.2.2 CHARACTERISTICS OF gp60 (PREPARED BY METHOD 1) BINDING TO IgG

In order to study ¹²⁵[I]-gp60 binding to IgG-IC, BSA/anti-BSA solid-phase IC was formed using immunoabsorbent purified IgG anti-BSA, where the number of molecules of IgG per well was determined. The addition of increasing amounts of ¹²⁵[I]-gp60 to solid-phase IC was associated with increasing binding to the wells in which 3.4×10^{10} molecules of IgG were bound per well (Fig. 4.2). When bound ¹²⁵[I]-gp60 was plotted on the ordinate against free ¹²⁵[I]-gp60 on the abscissa, the dose-response curve was concave towards the abscissa (Fig. 4.2) indicating that the binding was saturable, and at saturation there was 6.8×10^9 molecules of ¹²⁵[I]-gp60 bound per well. At saturation 1 molecule of gp60 was bound for every 5 available IgG molecules. In this experiment only 0.034% of the offered ¹²⁵[I]-gp60 was bound, but this increased

Figure 4.1:

Binding of 125 [I]-gp60 (monomer) to immunoglobulin isotypes.

- a) Wells were coated with 10 ng IgG (○—○), 5 ng IgG (△ △), 1 ng IgG (□—□), 10 ng IgM (■—■) or 10 ng IgA (▲—▲). Different amounts of 125 [I]-gp60 (1.3-20 ng) were added/well.
- b) Kinetics of 125 [I]-gp60 binding to solid-phase IgG. Wells were coated with 5 ng IgG, 20 125 ng [I]-gp60 was added to each well at time 0, and aspirated at times shown on the abscissa.

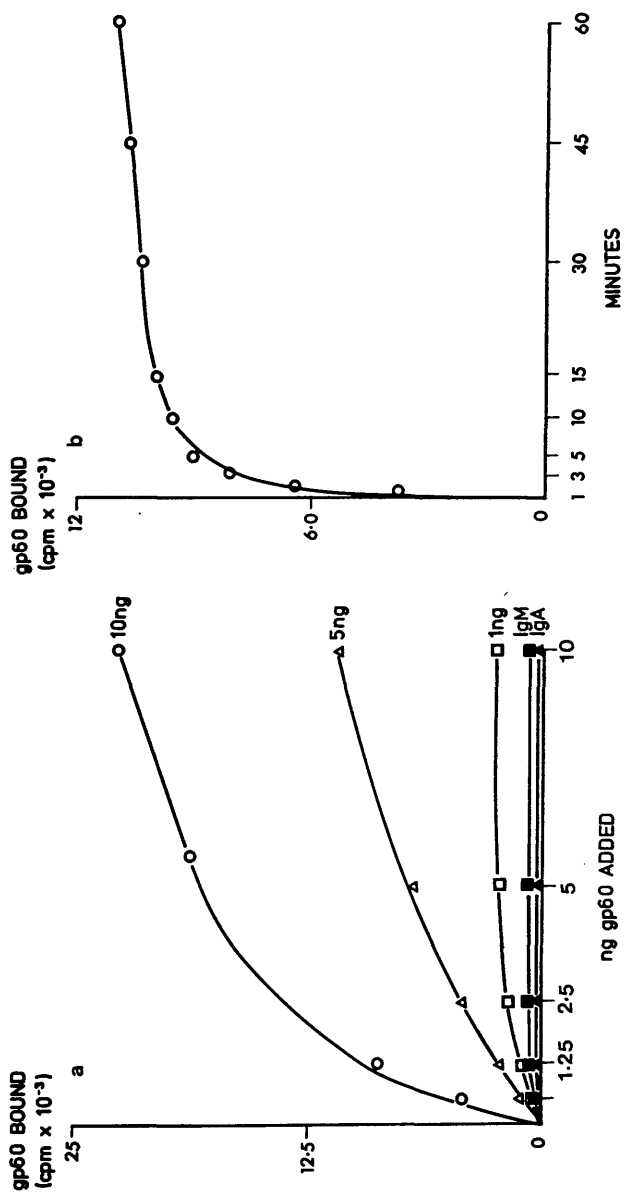
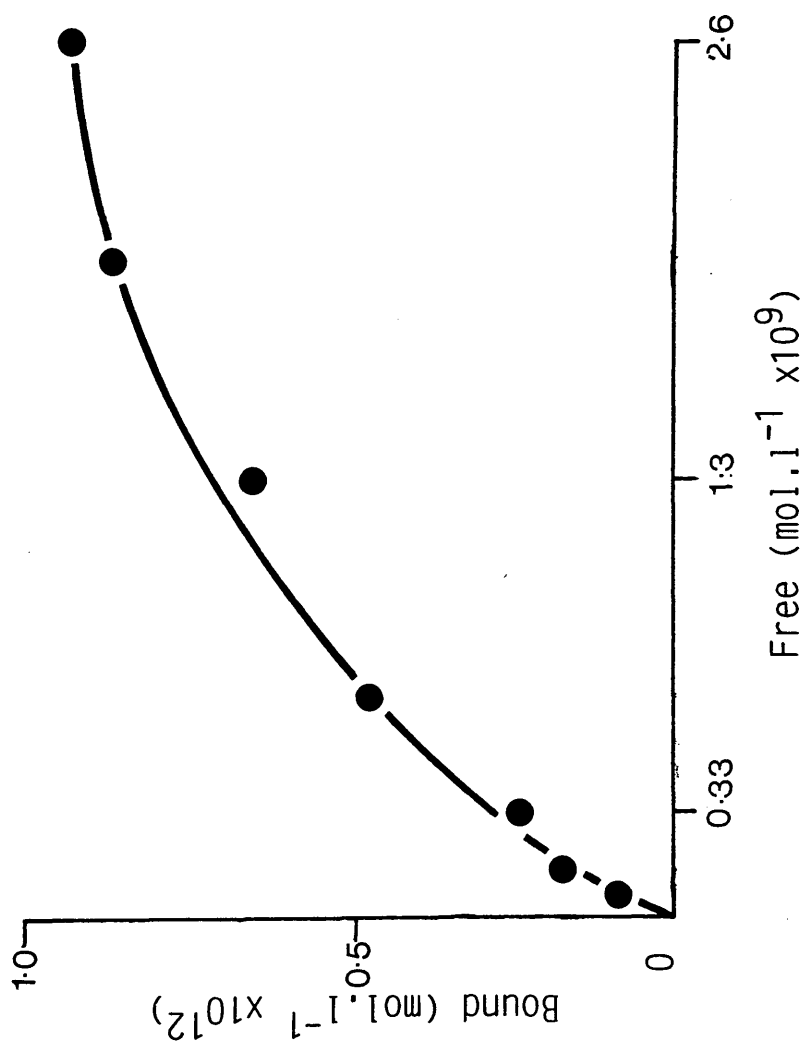


Figure 4.2:

Direct plot of ¹²⁵I]-gp60 monomer binding to
solid-phase BSA-IgG-anti-BSA IC. ¹⁰3.4x10
molecules of IgG were bound per well. Bound
¹²⁵I]-gp60 on ordinate plotted against Free
¹²⁵I]-gp60 on abscissa. Only 0.034% of offered
¹²⁵I]-gp60 was bound.



to 1.2% when the number of molecules of IgG per well was increased to 5.7×10^{12} .

To determine the affinity of gp60 binding to solid-phase IgG-IC, the data were analysed by Scatchard and double-reciprocal plots. Scatchard plot analysis in which the ratio of the number of molecules of 125 [I]-gp60 bound / the number of free 125 [I]-gp60 molecules was plotted against the number of 125 [I]-gp60 molecules bound per molecule of IgG, revealed a single regression line, indicating the presence of only a single class of binding sites for gp60 on the IgG molecule. The intercept on the abscissa indicated that at saturation 0.213 molecules of gp60 was bound per IgG molecule, giving a ratio of 1 molecule of gp60 to 5 molecules IgG (Fig. 4.3). The binding affinity of gp60 for IgG which was calculated as 9.4×10^8 1/mol. In double-reciprocal plot analysis (Fig. 4.4) where $1/B$ (the reciprocal of moles of bound 125 [I]-gp60 was plotted against $1/F$ (the reciprocal of moles of free 125 [I]-gp60), showed that the intercept on the ordinate which is the reciprocal of maximum attainable molar concentration of bound 125 [I]-gp60 was 0.23×10^{11} 1/mol. The affinity constant was calculated from the slope of the line which was equal to $1/nK$. In this case the affinity constant was calculated to be 9.4×10^8 1/mol.

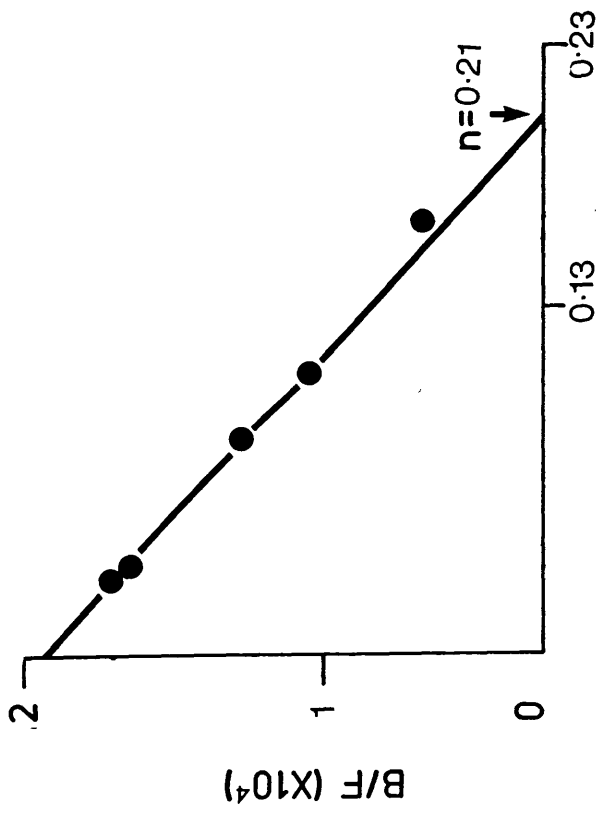
From Hill plot analysis (Hill, 1910) of the same data (Fig. 4.5), the Hill coefficient was equal to 0.992, indicating the non-cooperativity of the binding of 125 [I]-gp60 to IgG solid-phase IC. These results were reproducible, as in a total of nine experiments, the mean

Figure 4.3:

Scatchard plot analysis of data shown in figure 4.2.

B/F, number of molecules 125 [I]-gp60
bound/number of molecules of 125 [I]-gp60 free.

The intercept on the abscissa shows the number of molecules of 125 [I]-gp60 bound per IgG molecule (0.213). The slope of the line represents the affinity constant of 125 [I]-gp60 binding to IgG-IC which was calculated as 9.8×10^8 l/mol.



Molecules inhibitor bound per IgG molecule

Figure 4.4:

Double reciprocal plot analysis of ¹²⁵[I]-gp60 binding to IgG-IC.

1/B: reciprocal of moles of ¹²⁵[I]-gp60 bound

1/F: reciprocal of moles of ¹²⁵[I]-gp60 free

n: maximum attainable molar concentration of bound ¹²⁵[I]-gp60.

The slope of the graph equals the reciprocal of the affinity constant x n. The affinity constant was calculated as 9.36×10^8 l/mol.

I/B
($\text{l.mol}^{-1} \times 10^{-11}$)

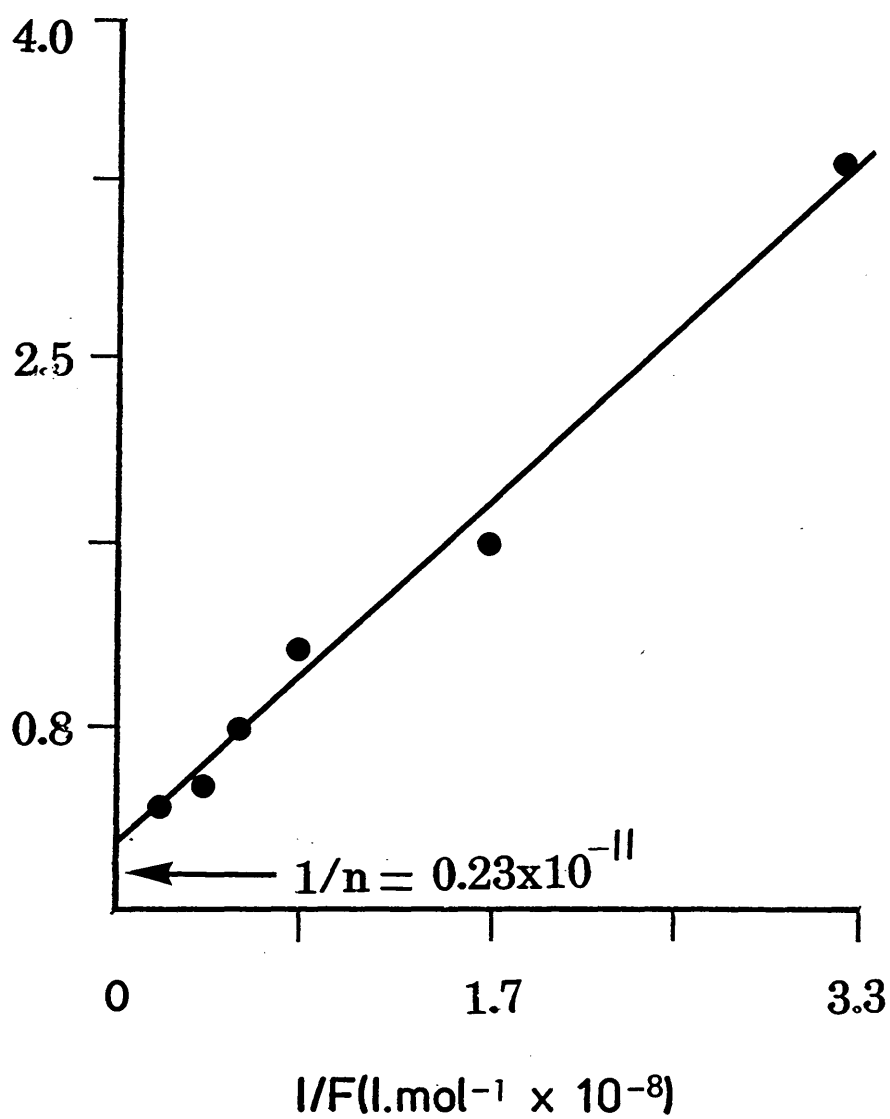


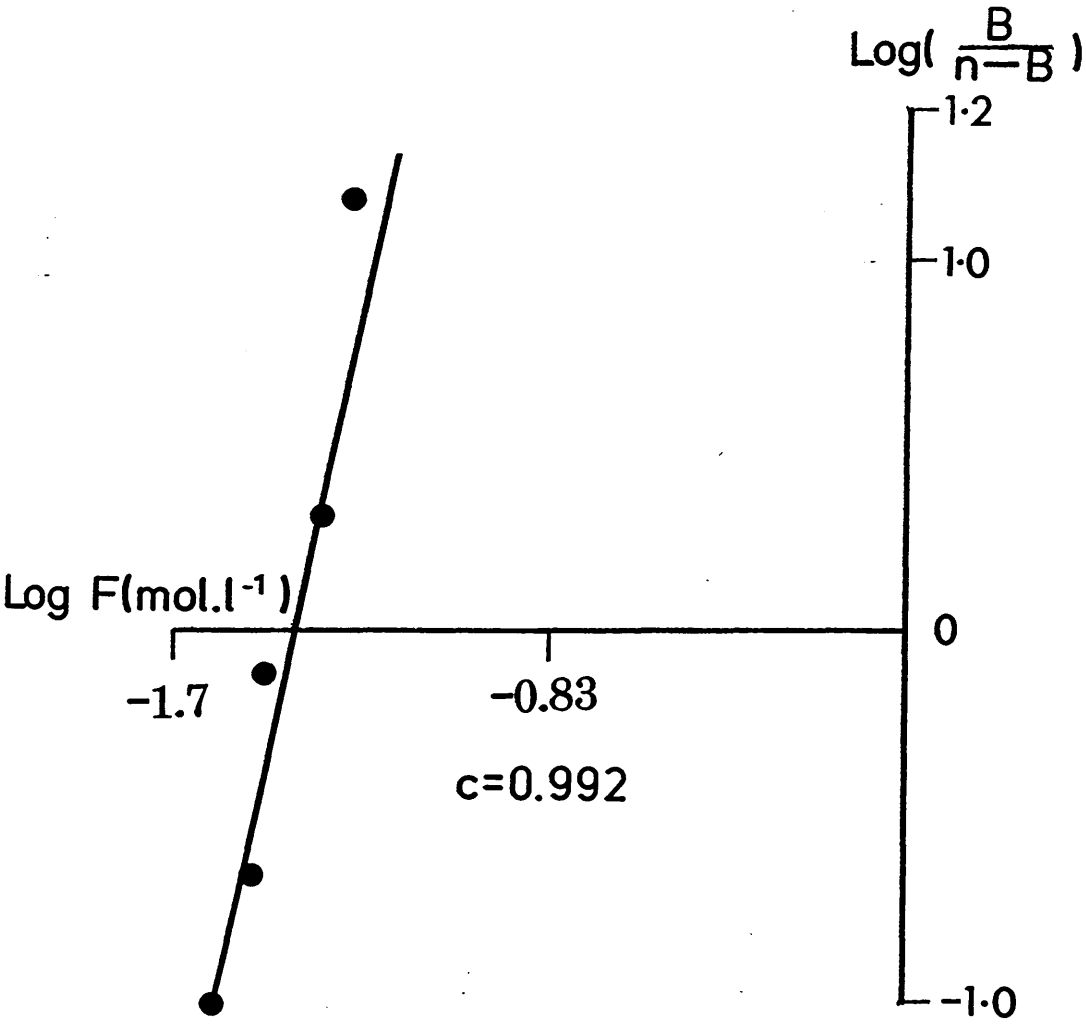
Figure 4.5:

Hill plot analysis of ^{125}I -gp60
monomer to IgG-IC.

B: number of moles ^{125}I -gp60 bound/l

F: number of moles ^{125}I -gp60 free/l

C: Hill constant (a measure of the slope of the
graph) which was calculated to be 0.992.



affinity constant K was calculated to be 9.48×10^8 l/mol (range 6.6 to 11.2×10^8 l/mol) and the mean Hill coefficient was 1.01 (range $0.91 - 1.12$).

4.2.3 COMPARISON OF THE BINDING OF DIFFERENT gp60 PREPARATIONS TO IgG

The binding to IgG of 125 [I]-gp60 prepared by each of the three methods was compared. Different concentrations of the 125 [I]-gp60 preparations were added to a series of microtitre wells containing solid-phase IgG-IC (4.1.11) with a known amount of bound IgG (8 ng/well). Direct plots and Scatchard plots showed that the characteristics of monomer binding (gp60 prepared by method 1) were similar to those determined previously (4.2.2), the affinity constant being 6.1×10^8 l/mol with one molecule of 125 [I]-gp60 bound per 5 molecules of IgG (Fig. 4.6). For the calculations involving the monomer, a molecular weight of 60 kD was assumed, while for those involving the binding of the polymer, the molecular weight was taken as 1000 kD. The binding characteristics of 125 [I]-gp60 prepared by method 2 showed higher affinity (1.7×10^9 l/mol) with one molecule of 125 [I]-gp60 binding for every 24 molecules of IgG (Fig. 4.7).

When the binding of 125 [I]-gp60 (prepared by method 3) was studied, it was found that one molecule of 125 [I]-gp60 bound per 26 molecules of IgG, and the affinity constant was 3.7×10^9 l/mol (Fig. 4.8). Each experiment in which the binding of the different 125 [I]-gp60 preparations to a fixed amount of bound IgG anti-BSA was determined, was repeated on three separate occasions. On each occasion

Figure 4.6:

Binding of ¹²⁵[I]-gp60 monomer to IgG-IC

a) Direct plot of the number of moles ¹²⁵[I]-gp60 bound plotted against the number of moles of ¹²⁵[I]-gp60 free.

b) Scatchard plot analysis of the data shown in (a). The affinity constant was found to be 6.1×10^8 l/mol and the number of moles of gp60 monomer bound per IgG mol at saturation was 0.26.

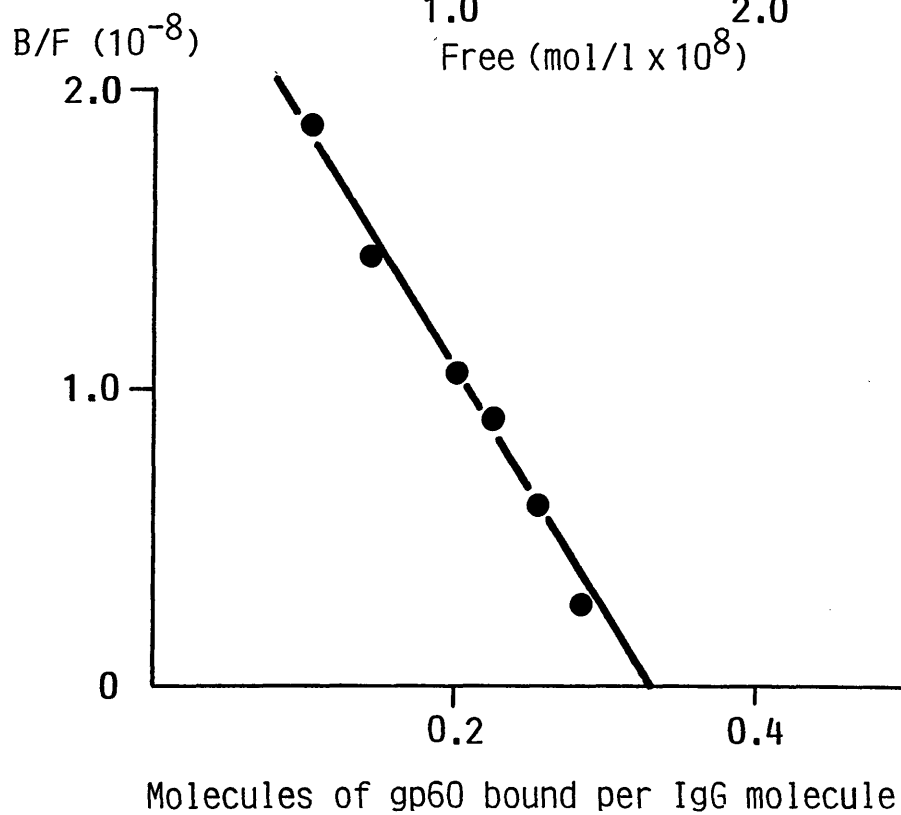
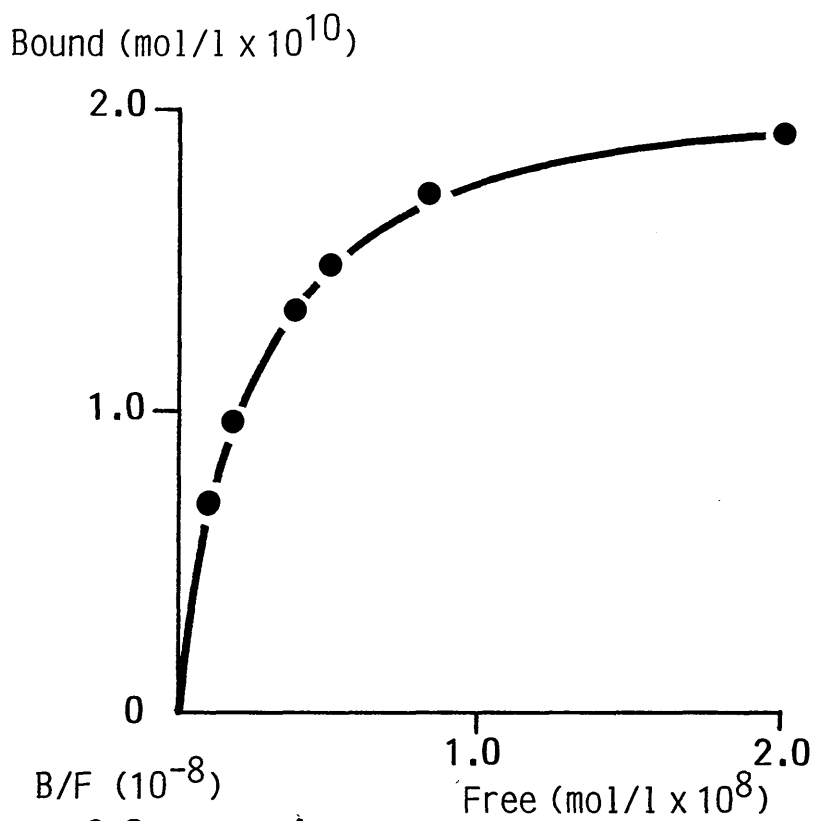


Figure 4.7:

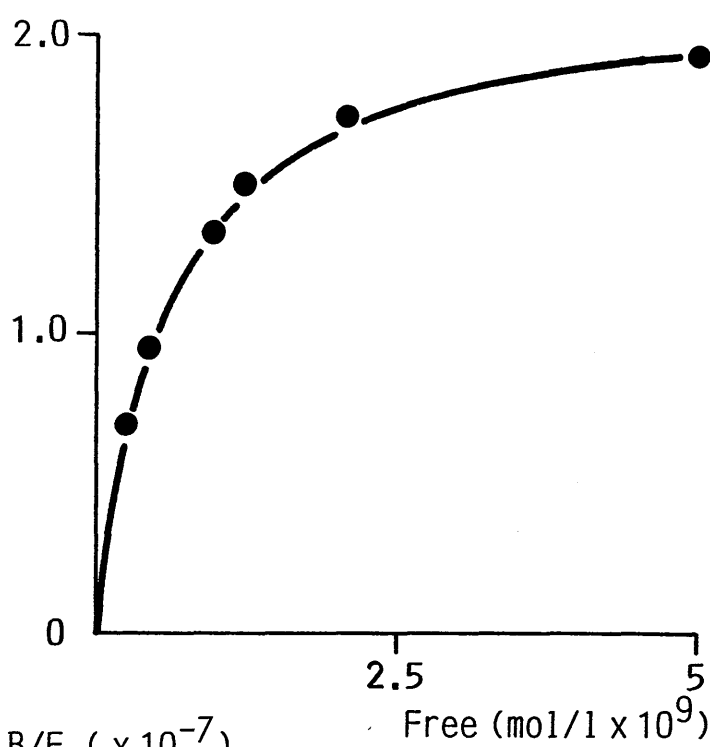
Binding of ¹²⁵[I]-gp60 polymer
(prepared by method 2) to IgG-IC.

a) Direct binding

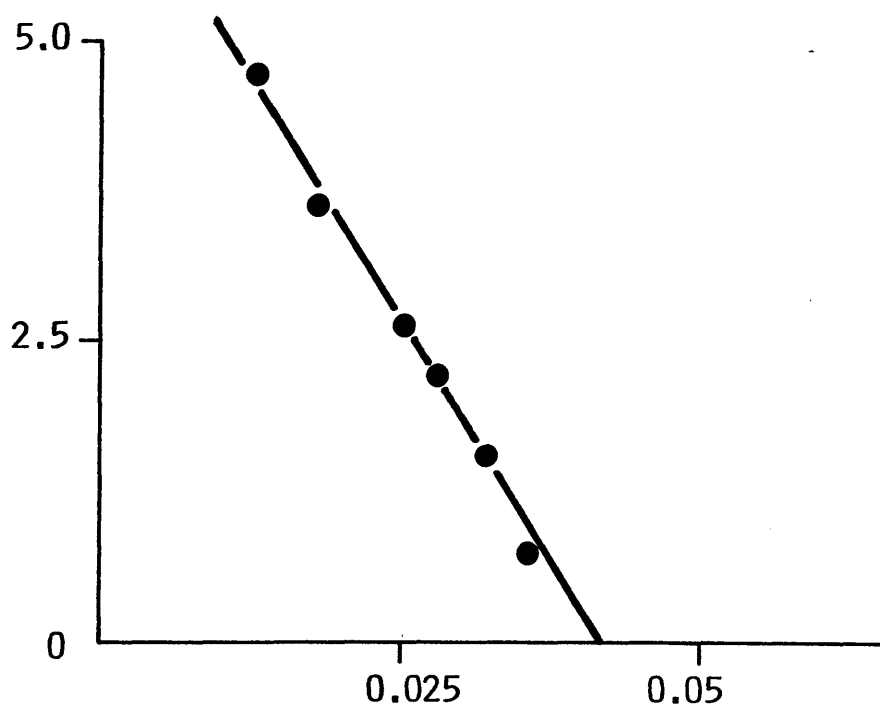
b) Scatchard plot analysis of the data in (a).

The affinity constant of ¹²⁵[I]-gp60 to IgG % IC
was found to be 1.7×10^9 l/mol and the number of
¹²⁵molecules of [I]-gp60 polymer bound per
molecule of IgG was 0.041.

Bound (mol/l $\times 10^{11}$)



B/F ($\times 10^{-7}$)



Molecules of gp60 bound per molecule of IgG

Figure 4.8

Binding of ¹²⁵I]-gp60 polymer prepared by
method 3 to IgG-IC.

a) Direct binding plot

b) Scatchard plot analysis of the data in (a)

The affinity constant was calculated as

⁹
 3.7×10^9 l/mol, and the number of molecules of
¹²⁵

[I]-gp60 polymer prepared by method 3 per IgG
molecule was found to be 0.039.

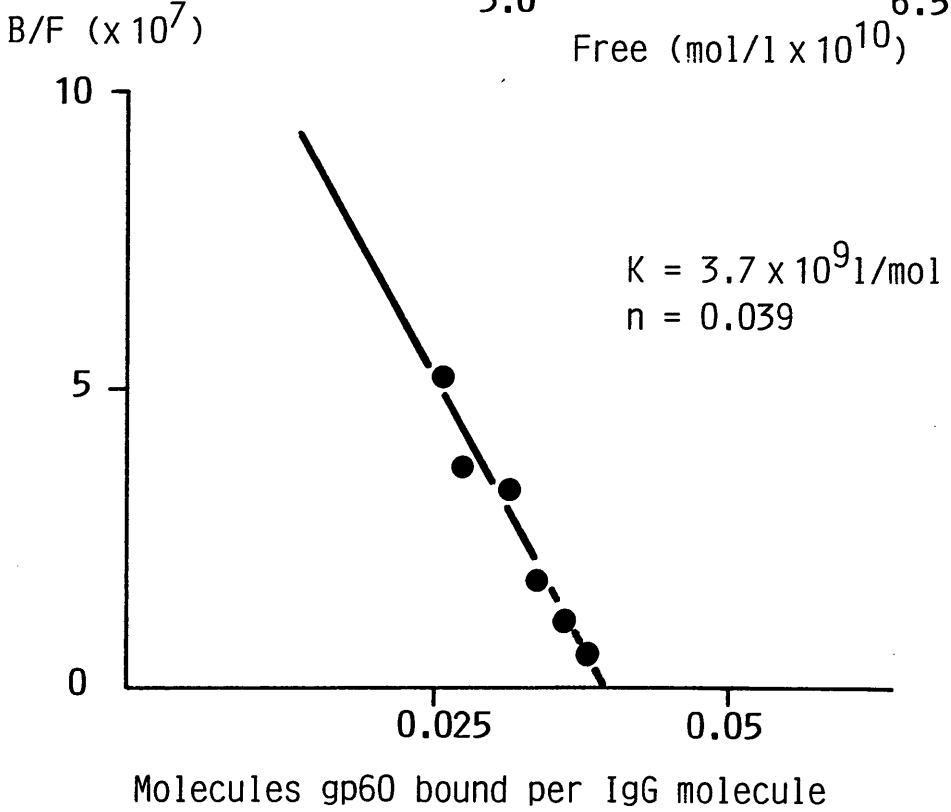
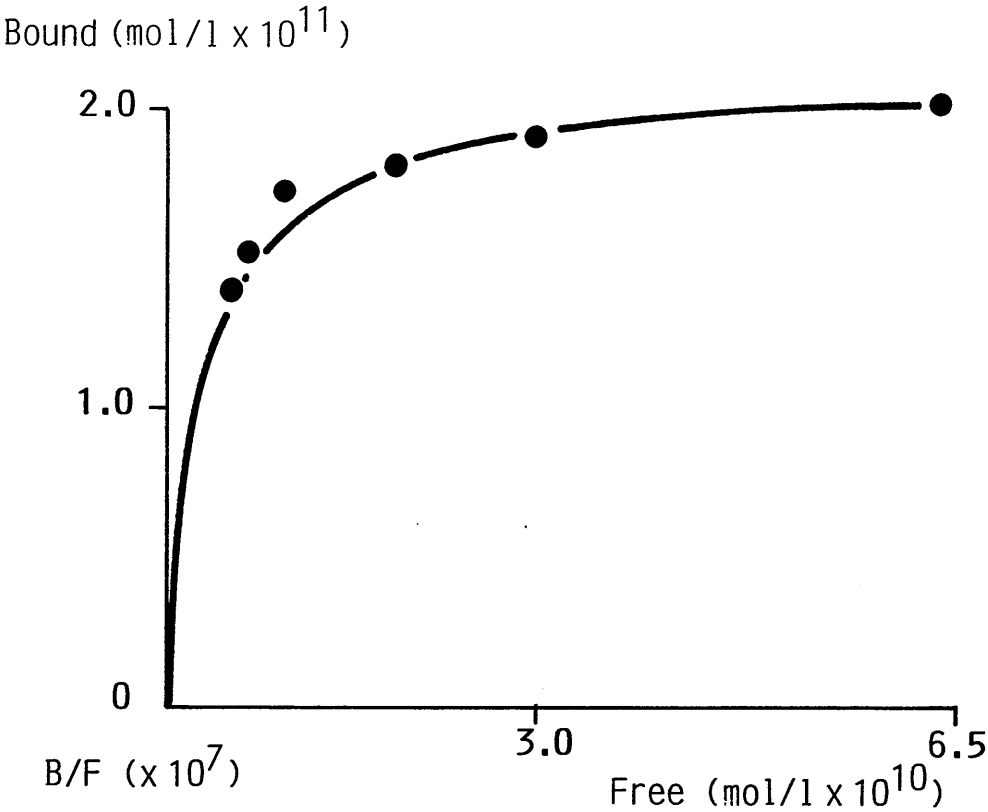


TABLE 4.1

K. Values of gp60 preparations for IgG-IC

gp60 preparation	K. Value l/mol			
	1ng IgG	8ng IgG	16ng IgG	32ng IgG
Method 1	9.2×10^8	6.1×10^8	4.6×10^8	1.3×10^9
Method 2	1.5×10^9	1.7×10^9	1.9×10^9	2.5×10^9
Method 3	3.9×10^9	3.7×10^9	3.6×10^9	-

similar results were obtained (see Table 4.1).

The binding studies were performed using four different amounts of bound IgG anti-BSA (1ng, 8ng, 32ng and 64ng bound IgG anti-BSA per well), and each set of experiments was repeated on three separate occasions. The results which are shown in Table 4.1 gave similar affinities to those in the experiments described. As saturation was not achieved when 64 ng IgG anti-BSA were bound per well, the ratio of molecules of gp60 bound per molecule of IgG could not be calculated. However, for the three lower amounts of bound IgG the molar ratios of ¹²⁵

[I]-gp60 bound per IgG were similar to those calculated in the first series of experiments.

4.2.4 LOCALISATION OF THE gp60 BINDING SITE ON IgG

The binding site for gp60 on the IgG molecule was localised from direct binding and competition inhibition studies with F(ab')₂, Fab and Fc fragments of normal human IgG. [I]-gp60 showed dose-dependent binding to solid-phase Fc, but there was no binding to Fab and very little binding of F(ab')₂. These results were confirmed by showing that Fc fragments were able to inhibit the binding of ¹²⁵[I]-gp60 to solid-phase IgG-IC in a dose-dependent fashion, while Fab and F(ab')₂ fragments had no effect (Fig. 4.9).

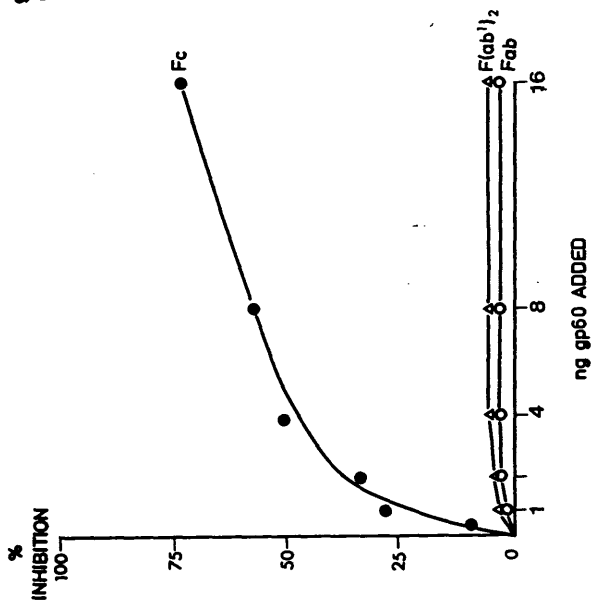
4.2.5 EFFECT OF IgG SUBCLASSES ON gp60 BINDING

In competition inhibition assays, IgG1 and IgG3 were more effective than IgG2 and IgG4 in reducing the binding of ¹²⁵[I]-gp60 to solid-phase IgG-IC (Fig. 4.10). ¹²⁵[I]-

Figure 4.9:

Binding of ¹²⁵[I]-gp60 monomer to IgG fragments.
a) Inhibition of ¹²⁵[I]-gp60 binding to solid-phase IC by different amounts of Fc (●—●), Fab (○—○) and F(ab') (Δ—Δ).
b) Direct binding of ²²⁵[I]-gp60 to wells coated with 10 ng Fc (●—●), 10 ng Fab (○—○) and 10 ng F(ab') (Δ—Δ).

a) COMPETITION



b) DIRECT BINDING

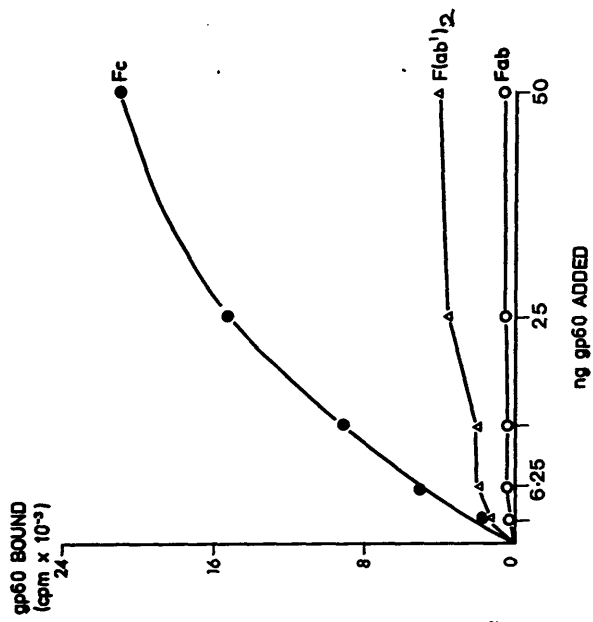
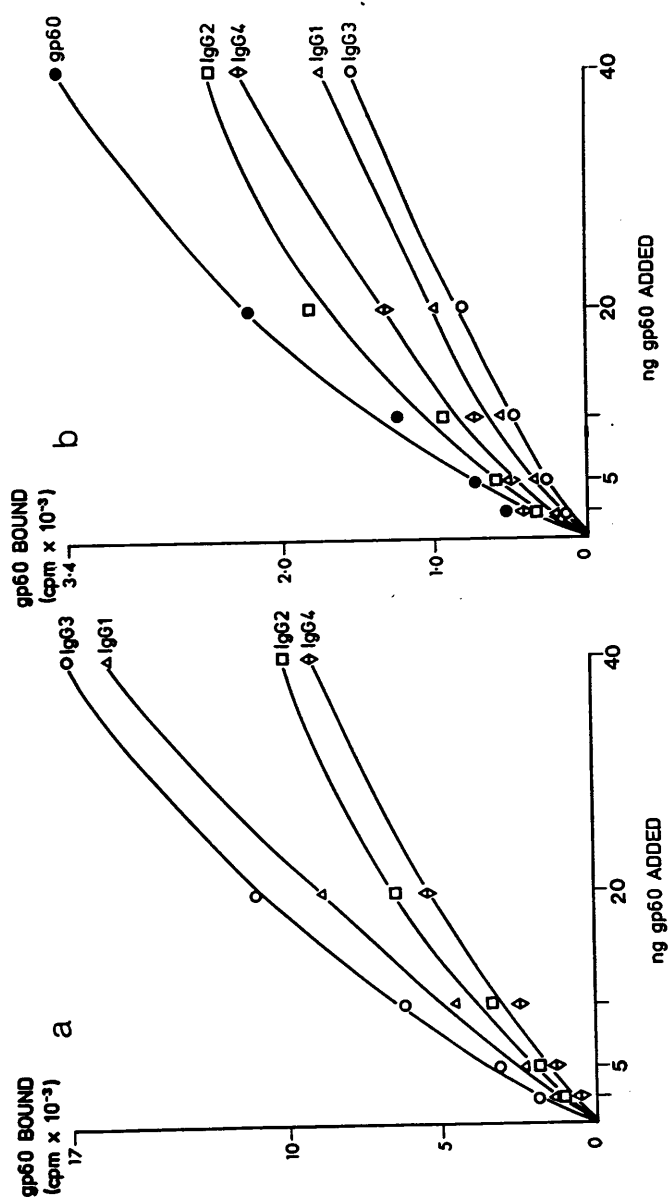


Figure 4.10:

Binding of ¹²⁵[I]-gp60 to IgG subclasses (●—●).
a) Direct binding. Different amounts of ¹²⁵[I]-gp60 were added to microtitre wells coated with 10 ng IgG1 (Δ-Δ), IgG2 (□-□), IgG3 (○-○) and IgG4 (◇-◇).
b) Inhibition of ¹²⁵[I]-gp60 binding to solid-phase IgG-IC by, 10 ng IgG1(Δ-Δ), IgG2 (□-□), IgG3(○-○) and IgG4 (◇-◇) were added simultaneously with different concentrations of ¹²⁵[I]-gp60 (2.6-40 ng) to microtitre wells coated with BSA-anti-BSA IgG solid-phase complexes.



gp60 bound more effectively to solid-phase IgG1 and IgG3 than to the IgG2 and IgG4 subclasses (Fig.4.10). Scatchard plot analysis of the direct binding of ¹²⁵[I]-gp60 to solid-phase IgG subclass proteins, showed that affinity of gp60 for each subclass was as follows: IgG1, 5.2×10^8 l/mol; IgG2, 2.3×10^8 l/mol; IgG3, 5.5×10^8 l/mol; and IgG4, 1.1×10^8 l/mol.

4.2.6 EFFECT OF Gp60 ON C1q BINDING

Unlabelled gp60 (4 - 32 ng) produced dose-dependent inhibition of the binding of ¹²⁵[I]-C1q (200 ng) to solid-phase IgG but did not affect its binding to solid-phase IgM (Fig. 4.11). Inhibition of ¹²⁵[I]-C1q binding to solid-phase IgG was also seen in the converse experiment in which a single dose of gp60 (32 ng) was incubated with different amounts of ¹²⁵[I]-C1q (25-200 ng).

In a second series of experiments, the effect of different amounts of unlabelled gp60 (4.2, 8.4 and 16.8 ng) on the binding of ¹²⁵[I]-C1q to solid-phase BSA/anti-BSA IgG-IC was studied. Again gp60 caused dose-dependent inhibition of the binding of ¹²⁵[I]-C1q to IgG.

Scatchard plot analysis of these studies showed that with increasing concentration of gp60. the apparent affinity of C1q to IgG-IC was reduced, but the number of binding sites was unaltered (Fig. 4.12). The affinity constant of C1q binding to solid-phase IgG-IC was calculated to be 1.1×10^8 l/mol, and in the presence of 4.2, 8.4 and 16.8 ng gp60 it was reduced to 9×10^7 l/mol, 6.9×10^7 l/mol and 5.6×10^7 l/mol respectively. In the

Figure 4.11

Effect of gp60 on ¹²⁵[I]-C1q binding to solid phase IgG or IgM.

- a) Unlabelled gp60 (4-32 ng) was added simultaneously with a fixed dose of ¹²⁵[I]-C1q (200 ng) to microtitre wells precoated with 10 ng IgG (●—●) or IgM (▲—▲).
- b) Unlabelled gp60 (32 ng) was added with different amounts of ¹²⁵[I]-C1q (12.5-200 ng) to microtitre wells precoated with 10 ng IgG (□—□), ¹²⁵[I]-C1q binding alone (●—●).
- c) Unlabelled gp60 was added with different amounts of ¹²⁵[I]-C1q (12.5-200 ng) to microtitre wells precoated with 10 ng IgM (□—□), ¹²⁵[I]-C1q binding alone (▲—▲).

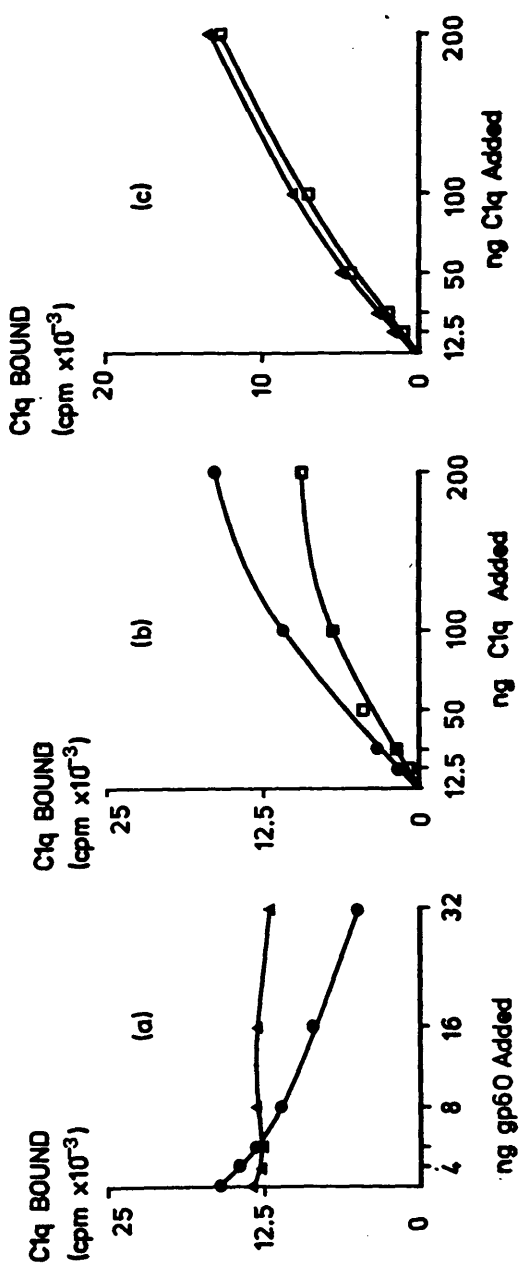


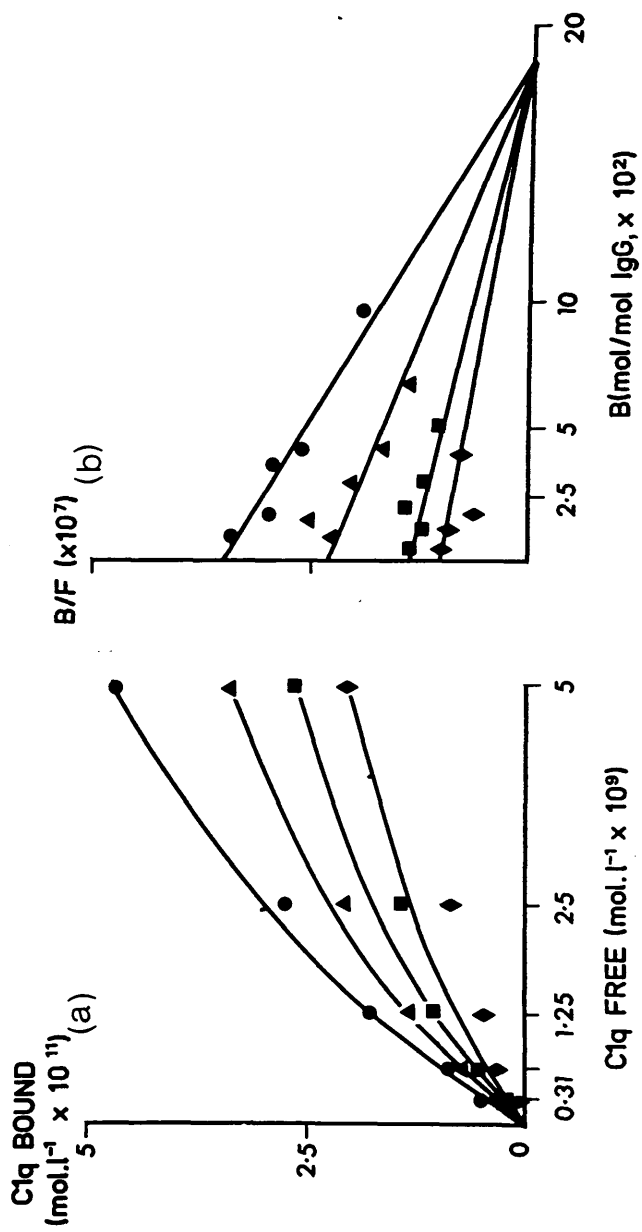
Figure 4.12:

Effect of gp60 on the binding of ¹²⁵[I]-C1q to solid-phase IC.

a) Direct binding of different amounts of ¹²⁵[I]-C1q (25-200 ng) to solid-phase BSA anti-BSA IgG (●-●) and in the presence of unlabelled gp60 4.2 ng (▲-▲), 8.4 ng (■-■) and 16.8 ng (◆-◆).

b) Scatchard plot analysis of data in (a).

The affinity constant of ¹²⁵[I]-C1q to IgG-IC (●-●) was calculated as 1.1×10^8 l/mol and in the presence of 4.2 ng gp60 (▲-▲), 8.4 ng gp60 (■-■) and 16.4 ng gp60 (◆-◆) it was reduced to 9×10^7 , 6.9×10^7 and 5.6×10^7 l/mol respectively. The number of molecules of ¹²⁵[I]-C1q bound per IgG molecule did not change in the presence of gp60.



converse experiment unlabelled C1q produced dose-
¹²⁵
dependent inhibition of [I]-gp60 binding to solid-phase
IgG-IC.

Analysis of these data by Scatchard plot showed that
the affinity constant of gp60 in the absence of C1q was
⁸
 7.5×10^8 l/mol, while in the presence of 25, 50 and 100 ng
⁸ ⁸
C1q it was reduced to 2.5×10^8 l/mol, 1.7×10^8 l/mol
⁷
and 9.2×10^7 l/mol respectively.

¹²⁵ [I]-gp60 did not bind to solid-phase C1q or to the
¹²⁵
solid-phase F(ab') fragments of C1q antiserum. [I]-C1q
¹²⁵ ²
and [I] F(ab') anti-C1q did not bind to solid-phase
²
gp60.

4.2.7 EFFECT OF gp60 ON THE BINDING OF RHEUMATOID FACTOR TO IgG

In a set of experiments designed to investigate the
effect of gp60 on rheumatoid factor binding to IgG,
unlabelled gp60 monomer produced dose-dependent inhibition
¹²⁵
of the binding of both [I]-IgM.RF (Fig.4.13), and
¹²⁵
[I]-F(ab') IgG-RF to solid-phase IgG-IC (Fig. 4.14).
²
Scatchard plot analysis showed an affinity constant for
⁶
IgM.RF as 5×10^6 l/mol which was reduced in the presence
⁶ ⁵
of 4.2, 8.4 and 16.8 ng gp60 to 1.8×10^6 , 9.8×10^5 , and
⁵
 5.7×10^5 l/mol respectively. The affinity constant of
⁶
F(ab') IgG-RF was found to be in the order of 2.1×10^6
² ⁵ ⁵ ⁵
l/mol, and was reduced to 5.5×10^5 , 3.9×10^5 and 2.6×10^5
l/mol respectively in the presence of gp60 at the same
concentrations. In the converse experiments both IgM-RF
and F(ab') IgG-RF were able to inhibit the binding of ¹²⁵
²

Figure 4.13:

Effect of unlabelled gp60 on 125 [I]-IgM-RF
binding to solid-phase IgG-IC.

- a) Direct binding plot of 125 [I]-IgM-RF alone
(• — •) and in the presence of 16.8 ng (◊ — ◊),
8.4 ng (◻ — ◻) and 4.2 ng (Δ — Δ) unlabelled gp60.
b) Scatchard plot analysis of data in (a).

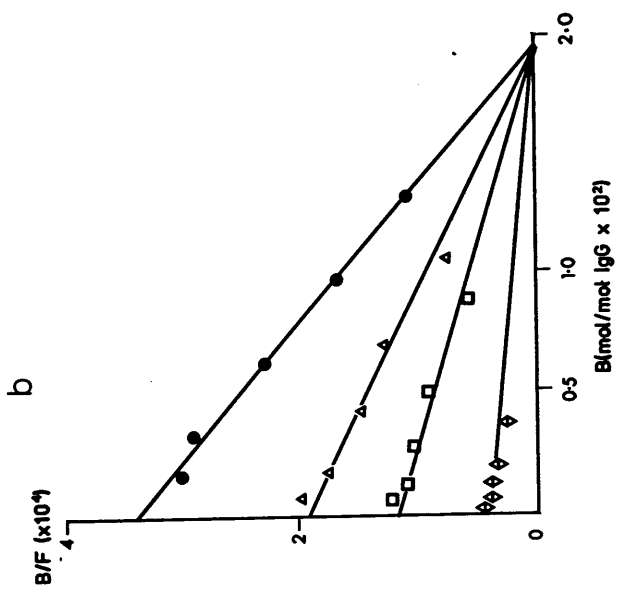
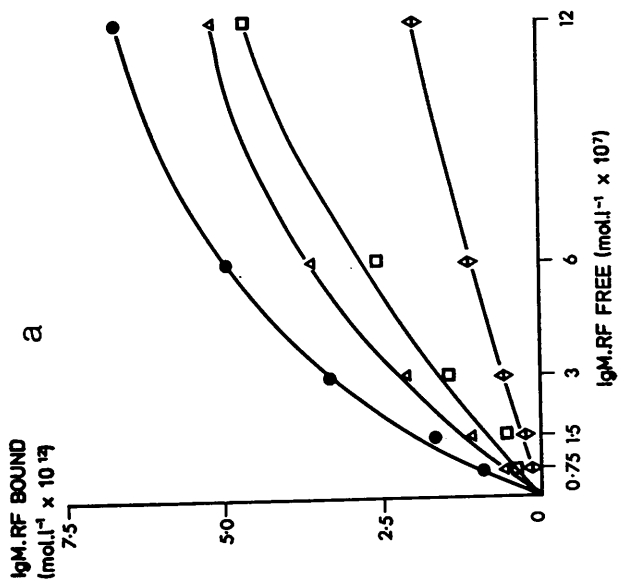
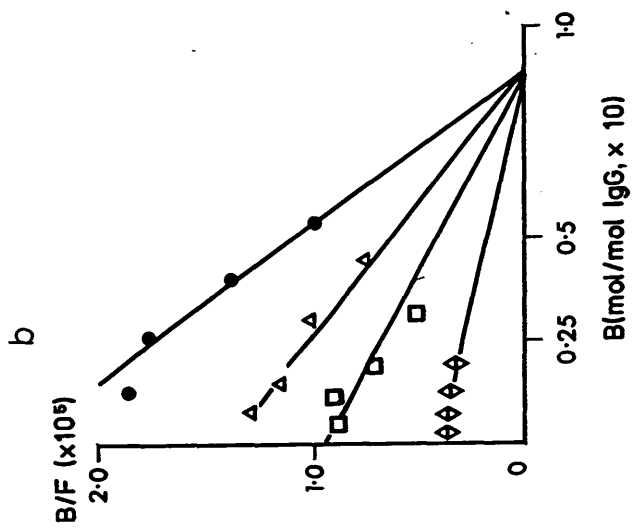
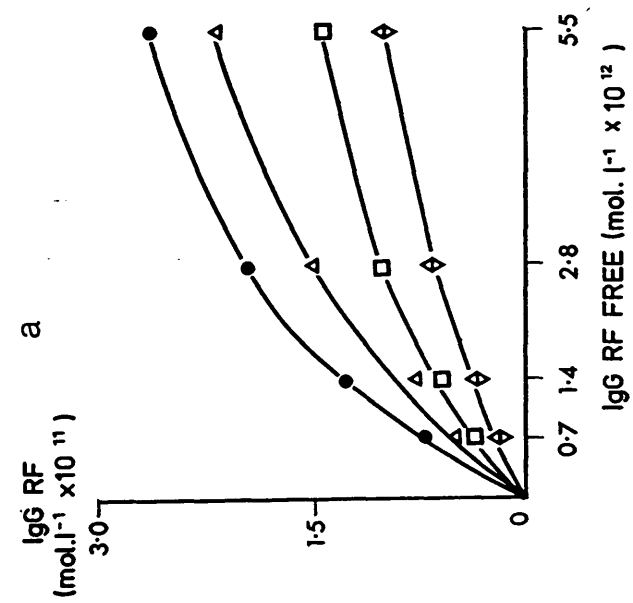


Figure 4.14:

Effect of gp60 on the binding of ^{125}I -F(ab')₂ IgG-RF to solid-phase IgG-IC.

a) Direct plot of ^{125}I F(ab')₂ IgG-RF to solid-phase IC alone (●—●), and in the presence of 16.8 ng (◊—◊), 8.4 ng (◻—◻) and 4.2 ng (Δ—Δ) unlabelled gp60.

b) Scatchard plot of data in (a).



[I]-gp60 to solid-phase IgG-IC in a dose-dependent fashion.

Both ²⁵[I]-IgM-RF and ¹²⁵[I]-F(ab')₂ IgG.RF did not bind to solid-phase gp60, and ¹²⁵[I]-gp60 monomer did not bind to solid-phase IgM-RF or F(ab')₂ IgG-RF.

4.2.8 EFFECT OF STAPHYLOCOCCUS AUREUS PROTEIN A ON BINDING OF gp60 TO IgG

Staphylococcus protein A produced dose-dependent inhibition of the binding of ¹²⁵[I]-gp60 to solid-phase IgG-IC.

DISCUSSION

The initial step of the gp60 purification procedure, affinity chromatography on IgG-Sepharose, indicated that the action of gp60 was most probably mediated by its binding to the antibody moiety of IC. Direct binding studies showed that gp60 binds to IgG but not to the IgM or IgA isotypes.

By studying the binding of ¹²⁵[I]-gp60 to solid-phase Fc, Fab and F(ab')₂ fragments of IgG, and by investigating the ability of these different fragments to inhibit the binding of ¹²⁵[I]-gp60 to solid-phase BSA/anti-BSA IgG-IC, it was clearly shown that the binding site for gp60 is located on the Fc piece of IgG. Further evidence to support this conclusion was provided by the experiments in which it was shown that C1q, IgM-RF, F(ab')₂ IgG-RF and Staphylococcus protein A were able to inhibit the binding of ¹²⁵[I]-gp60 to solid-phase IC. All four of these proteins have been shown to bind to the Fc piece of IgG. C1q binds to CH2 domain, IgM-RF and IgG-RF binds to either the CH2 or CH3 domains, while Staphylococcus protein A binds to both (Burton, 1985). These results emphasise the need to study the interactions of IgG, gp60 and these other Fc binding proteins in-vivo.

The selective binding of ¹²⁵[I]-gp60 to the IgG subclass myeloma proteins IgG1 and IgG3 is further evidence that the binding site for gp60 is located on the Fc piece of IgG. These two subclasses of IgG bind C1q, IgM-RF and IgG-RF better than IgG2 or IgG4 (Burton, 1985). IgG4 is thought not to bind to C1q or Fc receptors because the

conformation of the intact molecule prevents access of these proteins to the Fc piece (Burton, 1985), although the isolated Fc fragment of IgG4 can bind C1q (Isenman, Dorrington & Painter, 1975). The finding that gp60 showed some binding to IgG4 in these experiments is probably explained by the residual contamination of IgG4 with IgG3 (Bird. P., Personal communication).

The studies of the binding of ¹²⁵I]-gp60 to solid-phase IgG anti-BSA IC containing a known amount of IgG bound per well allowed important parameters of the interaction between gp60 and IgG to be determined. The affinity of the interaction between gp60 monomer and IgG was determined from Scatchard plots and double reciprocal plots, both of which gave similar results with a mean affinity of 9.48×10^8 1/mol (range $6.6 - 11.2 \times 10^8$ 1/mol). This value was lower than that obtained for the two polymeric forms of gp60, that prepared by method 2 had a mean value of 1.7×10^9 1/mol (range $1.1 - 2.3 \times 10^9$ 1/mol), while that prepared by method 3 was even higher, mean 3.7×10^9 (range $2.3 - 5.1 \times 10^9$ 1/mol). The higher affinity constants for the polymeric forms can be explained on the basis of the increased number of binding sites for IgG per molecule of polymer compared with the monomer.

The significantly higher binding affinity for IgG of ¹²⁵I]-gp60 prepared by method 3 compared with ¹²⁵I]-gp60 prepared by method 2 is most likely due to partial denaturation of gp60 during its elution from IgG-Sepharose by 2M guanidine hydrochloride. The alternative explanation that a high affinity fraction of gp60 remained bound to the IgG-Sepharose is improbable.

At saturation with monomeric gp60 only 0.2 molecules of gp60 were bound for each molecule of IgG in the solid-phase IC. As a similar result was obtained when different amounts of IgG were bound to the wells, it is unlikely that steric hindrance accounts for the observation that saturation of gp60 binding to IgG is achieved only when one fifth of the available IgG binding sites are occupied.

It could be argued that rabbit C1q or other IgG binding proteins were co-purified with the specific antibody. However, this is unlikely as the purification procedure involved affinity chromatography on BSA-Sepharose followed by protein A-Sepharose chromatography, and C1q could possibly bind to IgG in both procedures (particularly the former), analysis of the antibody by SDS-PAGE did not reveal any obvious contaminants. It is therefore likely that only 20% of the rabbit IgG anti-BSA molecules are capable of binding gp60. The possible explanations for this include:-

- 1) Damage to 80% of the molecules during purification.
- 2) Following binding of antibody to antigen the conformation of 80% of the antibody molecules may prevent gp60 binding.
- 3) The 20% of rabbit IgG molecules represents the only subclass or a mixture of subclasses of IgG which can bind gp60.

Damage to 80% of the IgG molecules could occur during the purification procedure as an acid elution step (glycine-HCl, pH 3.0) was used for the purification of the antibody. However, I consider this degree of damage to be unlikely, as it would have been described by authors

studying C1q binding or C1 activation, or IgG-Fc receptor interactions.

It has been suggested that the angle of the Fab arms of IgG can vary between 0° and 180° (Valentine and Green, 1967). Those molecules in which the angle was less than 60° could not fix complement, while those in which the angle was between 90° and 180° possessed complement fixing ability (Hyslop et al, 1970). These data show that in addition to aggregation for the complement activation to occur, and by implication the binding of C1 to IC, IgG molecules must acquire a specific configuration that is determined by the angle of the Fab arms. If the configuration is important for C1 binding, it could be equally important for the binding of gp60 to IgG, as gp60 and C1q competitively inhibit the binding of each other. Thus it could be argued that only 20% of the IgG anti-BSA in the solid-phase IC were in suitable configuration for gp60 binding to occur. The observation that at saturation the molar ratios of gp60 to IgG were independent of the number of bound IgG molecules argues against this explanation. In the experiments in which these molar ratios could be calculated, the amount of IgG bound per well varied from 1-32 ng. It is probable that over this range the angle of the Fab arms would have altered significantly and a change in the molar ratio of gp60 to IgG would have been observed.

Evidence to support the third possibility comes from experiments of Doekes et al (1985), who showed that both aggregated and monomeric human IgG was between two and five

times as effective as the rabbit IgG equivalents in terms of binding human C1. Although these authors did not perform solid-phase studies, their data supported the view that only a proportion of rabbit IgG molecules can bind C1. If this is the case, then it is possible that only a proportion of rabbit IgG molecules will bind gp60. Other evidence to support the third explanation has been provided by Hughes-Jones and Gardner (1978), who showed that in fluid-phase DNP-rabbit anti-DNP IC system only one molecule of C1q bound for every 10-14 molecules of IgG. On balance, the third possibility appears to provide the most reasonable explanation for my findings.

The binding studies with both preparations of polymeric gp60 showed that at saturation the molar ratios of gp60 to IgG were similar (mean 1:24 for preparation 2 and mean 1:26 for preparation 3). The ratio was slightly higher for the lowest amount of bound IgG (1:27 and 1:30 respectively).

These ratios were derived by using a molecular weight of 1000 kD for gp60, are higher than might be expected from the binding data for monomeric gp60 (1:5), assuming that polymeric gp60 is composed of 16 monomeric subunits. The most probable explanation is that only a minority of the available binding sites on gp60 have been bound to IgG in these experiments.

The finding that the affinity constant of ¹²⁵[I]-gp60 to polyclonal IgG was higher than for its binding to IgG1 or IgG3, is probably explained on the basis of species and technical differences of the assays used. For the studies of the interaction of gp60 with polyclonal IgG, rabbit

antibody was used in the form of an IC so that all the sites were presented for optimal interaction with Fc binding proteins. In contrast, in the studies with immunoglobulin subclasses, human myeloma proteins bound directly to the wells were used. This binding is entirely random so it is probable that some Fc pieces were able to bind to gp60 while others may have been unable to do so. Of those molecules which were able to bind gp60, it is likely that the orientation of some was such that the affinity of the interaction was reduced.

The failure of ¹²⁵[I]-gp60 to bind to solid-phase C1q and ¹²⁵[I]-C1q to bind to solid-phase gp60 confirms that gp60 acts on the IgG antibody moiety of IC and not on the C1 component of complement. Its failure to bind to solid-phase F(ab') fragments of IgG anti-C1q and the failure of ¹²⁵[I]-F(ab')₂ anti-C1q to bind to solid phase gp60 shows that it is not an altered form of C1q. The lack of binding of gp60 to IgM-RF or F(ab') fragments of IgG-RF and the failure of ¹²⁵[I]-IgM-RF and ¹²⁵[I]-F(ab')₂ IgG-RF to bind to solid-phase gp60 show that gp60 is not an altered fragment of the IgG heavy chain, as such a fragment would probably be detected by these autoantibodies, which react with IgG (Carson, 1984).

The affinity of C1q for IgG was calculated to be ⁸between 1.1 and 3.1x10⁸ l/mol which is in agreement with results of other authors (Hughes-Jones and Gardner, 1978), and is close to that calculated for monomeric gp60, but significantly lower than that calculated for polymeric gp60 which had been prepared by method 3. The mean

concentration of gp60 and C1q in normal serum are 34 ug/ml (see chapter 5) and 75 ug/ml (Tenner, Lesavre & Cooper, 1981) respectively. Thus their relative molar concentrations must be similar as their molecular weights are 1000 kD and 410 kD respectively.

If this is the case, it is difficult to understand how complement activation occurs, as the high affinity of gp60 for IgG would prevent C1 binding and activation to IgG containing IC. Perhaps the presence of IgM in the IC would allow complement activation to occur. Alternatively, gp60 activity in vivo may be regulated by another plasma constituent.

The studies on the interaction of gp60 and C1q with IgG suggest that once IgG antibody binds to antigen, the progression to a large tissue damaging lattice could well depend on the relative serum concentrations of gp60 and C1q. Relatively high levels of gp60 would favour precipitation, while relatively low levels (with respect to C1q) would favour PIP and the retention of IC in solution in a non-phlogistic form. Thus the outcome of the interaction of IgG antibody with antigen, with respect to whether the IC would remain soluble or form an insoluble lattice, could well depend upon the relative plasma concentrations of C1q and gp60 and their relationship to inhibition of PIP.

CHAPTER 5

THE EFFECT OF GP60 ON THE ACTIVATION OF THE CLASSICAL PATHWAY OF COMPLEMENT

INTRODUCTION

In the previous chapter it was shown that gp60 bound to the Fc piece of IgG but not to the IgA or IgM isotypes. Furthermore, it was shown that gp60 competitively inhibited the binding of C1q to IgG. PIP is dependent upon an intact classical pathway (Schifferli et al, 1982; Naama et al, 1984). The important events are the binding of the C1 macromolecule to IC, which slows the rate of immune precipitation (Shifferli & Peters, 1983; Naama et al, 1984), subsequent C1 activation, binding of C4b to IC, C3 convertase formation followed by C3 activation and binding of C3b to IC (Naama et al, 1984; 1985). The binding of C3b is thought to prevent the Fc-Fc interactions which facilitate immune precipitation (Moller, 1979; Moller & Steensgaard, 1979).

In this chapter experiments designed to investigate the effect of gp60 on classical pathway activation are reported. The results of these experiments show that gp60 prevents C1 activation, as a consequence of which C3 convertase formation is limited and C3 activation is reduced.

METHODS

5.1.1 SERUM SAMPLES

Venous blood was collected from 12 normal healthy laboratory personnel (6 males and 6 females) and 21 patients (9 male and 12 female) with definite or classical rheumatoid arthritis (Ropes et al, 1959). The serum was collected and stored as described in 2.16.

5.1.2 MEASUREMENT OF C1s-C1-INHIBITOR COMPLEX BY ELISA (Nilsson and Bock, 1985)

Microtitre plates were coated with 5 µg/ml IgG fraction of anti-C1s in 10 mM carbonate/bicarbonate buffer pH 9.6 (100 µl/well) overnight at 4 °C. The first two rows of the plate were coated with 5 µg/ml IgG anti-C1-inhibitor. The wells were washed five times in PBS-Tween (PBS containing 0.05% v/v Tween-20), and blocked with gelatin (0.1% w/v in PBS) for one hour at room temperature. After another five washes in PBS-Tween, doubling dilutions of a C1-inhibitor standard in PBS-EDTA-Tween (PBS-Tween containing 10 mM EDTA) were added to the first two rows of wells, while the diluted test samples in PBS-EDTA-Tween were added to the rest of the wells. After incubation for one hour at room temperature, the wells were washed five times in PBS-Tween, and 1/1000 dilution of IgG biotin-conjugated anti-C1-inhibitor was added. The incubation was allowed to proceed at room temperature for a further hour, after which the wells were washed again five times in PBS-Tween, and a 1/1000 dilution of avidin-HRP in PBS-EDTA-Tween was added. After one hour at room

temperature, the wells were washed as described earlier, and developed using the same conditions described in 3.1.26.

A standard curve was plotted using the absorbance values of the standard dilutions against their C1-inhibitor concentrations. The concentrations of C1s-C1-inhibitor complex in the test samples were determined by interpolation from the standard curve, and results were expressed as μg C1-inhibitor/ml. In all experiments a positive control of IC-activated serum was included.

5.1.3 MEASUREMENT OF C3-PROPERDIN COMPLEX BY ELISA (Mayes et al, 1984)

The same procedure to that described in 5.1.2 was used to coat wells with anti-properdin antibodies and biotin-conjugated anti-C3 was used as the detecting antibody. The sequence of incubation and washing steps was identical to that used in the C1s-C1 inhibitor assay. In this case the standard curve was prepared in wells coated with anti-C3 antibody, and the results were expressed as μg C3/ml.

5.1.4 MEASUREMENT OF C5b-9 COMPLEXES BY ELISA (Gawryl et al, 1986)

The same procedure to that described in 5.1.2 was used to coat wells with anti-C9 antibody, and biotin-conjugated anti-C5 was used as the detecting antibody. The sequence of incubation and washing steps was identical to that used in the C1s-C1 inhibitor assay. In this case the standard curve was prepared in wells coated with anti-C5 antibody, and the results were expressed as μg C5/ml.

5.1.5 C3a AND C4a MEASUREMENT BY RADIOIMMUNOASSAY

C3a and C4a were measured using commercially available radioimmunoassay kits. The test samples and standards were diluted in 0.15M NaCl and 100 μ l of each diluted sample or standard was transferred to a series of tubes (12x75mm). An equal volume of the precipitating reagent was added to each tube, and after mixing, the tubes were allowed to stand at room temperature for one hour. The precipitate (containing native C3 and C4) was separated by centrifugation (2000 g for 15 minutes at 2 °C), and 100 μ l of supernatant was transferred to a second tube containing 50 μ l assay buffer, followed by the addition of 50 μ l ¹²⁵[I]-C3a or ¹²⁵[I]-C4a, and 50 μ l antiserum to C3a or C4a. The tubes were incubated for 30 minutes at room temperature, and then 50 μ l of the secondary antibody were added. After mixing, the tubes were incubated at room temperature for 30 minutes before 2 ml isotonic saline was added to each tube. Following centrifugation (2000 g for 10 minutes at 4 °C), the supernatant was aspirated and the tubes were inverted over a Whatman filter paper until they were dry. The radioactivity of the pellets was counted in an automatic gamma counter.

Controls included:

- 1) Total input (50 μ l buffer, 50 μ l ¹²⁵[I]-C3a or 50 μ l ¹²⁵[I]-C4a,
- 2) Non-specific binding without first antibody (50 μ l buffer, 100 μ l saline and 50 μ l ¹²⁵[I]-C3a or 50 μ l ¹²⁵[I]-C4a, 50 μ l second antibody).

3) Zero standard (50 μ l buffer, 50 μ l saline, 50 μ l ¹²⁵[I]-C3a or 50 μ l ¹²⁵[I]-C4a, 50 μ l anti C3a or anti C4a, 50 μ l secondary antibody).

A series of standards of known C3a or C4a concentrations was included in each run.

Each assay was performed in duplicate and the results were calculated by averaging the c.p.m. for each replicate and subtracting the average non-specific binding from all the tubes except total input.

The percentage of bound isotope for each sample or standard was calculated using the formula:

% bound/Zero standard =

$$\frac{\text{Standard or test c.p.m.} - \text{Background c.p.m.}}{\text{Zero standard c.p.m.} - \text{Background c.p.m.}}$$

A standard curve was plotted on log-logit graph paper, using the percent bound/zero standard as a function of the logit and one as a function of the logarithm concentrations of C4a des Arg. or C3a des Arg. The concentration of anaphylatoxins in the samples were determined directly from the standard curve.

5.1.6 PREPARATION OF ANTIBODY-SENSITISED SHEEP ERYTHROCYTES (EA)

Sheep erythrocyte suspension was standardised as described in 2.15. The standardised cell suspension was mixed with an equal volume of antiserum (IgG or IgM antibody) to sheep erythrocytes (diluted to the maximum sub-agglutinating dose) in 0.01M EDTA-GVB, and incubated in a shaking water bath at 37 °C for 30 minutes. The EAs were then pelleted by centrifugation (2000 g for 5 minutes

at 4 °C), washed three times in 0.01M EDTA-GVB⁺⁺ and resuspended to the original volume in GVB⁺⁺. The EA preparation was stored at 4 °C until used.

5.1.7 PREPARATION OF EAC1 (Ruddy & Austen, 1967)

EAs were washed three times in GVB⁺⁺, and resuspended to 1x10⁸ cell/ml in DGVB⁺⁺ (2.16). The EA suspension (5 ml) was incubated with an equal volume of C1 (100 units/ml in DGVB⁺⁺) for 15 minutes in a shaking water bath at 37 °C. The EAC1 cells were pelleted by centrifugation (2000 g for 5 minutes at 4 °C), and resuspended to the original volume in DGVB⁺⁺. EAC1 preparations were used immediately in the measurement of C4 haemolytic activity.

5.1.8 PREPARATION OF RAT-SERUM-EDTA (C-RAT)

Rat serum was obtained as described in 2.16, and diluted 1/30 in 40 mM EDTA-GVB⁺⁺. The diluted serum was kept on ice and used immediately.

5.1.9 PREPARATION OF EAC4

Frozen EAC4 were kindly supplied by Dr. E. Holme. An aliquot of frozen EAC4 in a 15 ml Falcon tube was thawed at 37 °C in a shaking water bath using the following procedure: Two hundred and thirty microlitres (230 ul) D5OS was added to the cells drop-wise with shaking and incubated for 5 minutes at 37 °C. Mannitol-GVB⁺⁺ was added drop-wise with shaking in three stages involving the addition of 1, 2 and 8 ml respectively. Between each stage the cells were incubated for 2 minutes at 37 °C. After the third addition

of mannitol-GVB⁺⁺, the cells were transferred to a 50 ml Falcon tube and incubated for 5 minutes at 37 °C. DGVB⁺⁺ was then added drop-wise with shaking in four stages with incubation periods of five minutes between each stage. In the first stage 5 ml DGVB⁺⁺ were added, whereas in the subsequent three stages the volume was 10 ml. After the final addition of DGVB⁺⁺, the cells were incubated for 10 minutes at 37 °C and then centrifuged (2000 g for 5 minutes at 2 °C), washed three times in DGVB⁺⁺ and the cell count adjusted to 1×10^8 /ml in DGVB as described in 2.15

5.1.10 C4 HAEMOLYTIC ASSAY (Whaley, 1985)

Test sera were serially diluted in DGVB⁺⁺ (1/10000, 1/20000, 1/40000, 1/80000). One hundred microlitre (100 µl) of each dilution was incubated with equal volume of EAC1 at 37 °C for 15 minutes, followed by the addition of 100 µl guinea pig C2 (50 units/ml in DGVB⁺⁺), and the incubation continued at 30 °C for another 15 minutes to form EAC142. After the addition of 300 µl C-rat to each tube, and a further incubation at 37 °C for one hour, the reaction was terminated by the addition of 2 ml ice-cold saline to each tube.

Following centrifugation (2000 g for 5 minutes at 2 °C) to pellet unlysed cells, the optical density of the supernatants was measured at 414 nm. Controls of the assay included:

- 1) Cell blank (500 µl DGVB⁺⁺, 100 µl EAC1)
- 2) Reagent blank (100 µl DGVB⁺⁺, 100 µl EAC1, 100 µl C2, 300 µl C-rat)
- 3) 100% lysis (same as reagent blank but with the

substitution of water for saline at the end of the assay).

The control tubes were processed in an identical manner to test tubes. The results were calculated using the formula:

$$\% \text{ lysis} = \frac{\text{O.D sample} - \text{O.D reagent blank.}}{\text{O.D 100\% lysis} - \text{O.D reagent blank.}} \times 100$$

The number of haemolytic sites (Z) were calculated using the equation $Z = \ln (1 - y)$ where y = the preparation of cells lysed.

The concentration of units of C4 haemolytic activity was calculated as described (Whaley, 1985). Results of complement activation assays were expressed as % C4 consumption which was taken as % of haemolytic units which were consumed.

5.1.11 C2 HAEMOLYTIC ASSAY (Whaley, 1985)

Samples were serially diluted (1/1000 to 1/32000) in ⁺⁺ DGVB^o, and a 100 μ l was warmed to 30 C in a water bath. At timed intervals, a 100 μ l of EAC14 cells were added, and incubated for 2 minutes (T-max time), followed by the addition of 300 μ l C-rat^o, after which the tubes were transferred to a 37 C water bath in which the incubation was allowed to proceed for one hour. The reaction was terminated by the addition of 2 ml saline to each tube and centrifugation (2000 g at 4 C for 10 minutes), after which the O.D at 414 nm of the supernatant was measured. Control for the assay included:

1. Cell blank (400 μ l GVB⁺⁺, 100 μ l EAC14)
2. Reagent blank (100 μ l DGVB⁺⁺, 100 μ l EAC14, 300 μ l C-rat)

3. 100% lysis (Same as reagent blank but with the substitution of water for saline at the end of the assay.)

The control tubes were processed in an identical fashion to the test tubes. The results were calculated as for the C4 assay.

5.1.12 ANTISERA

The IgG fractions of anti-gp60 or of normal rabbit (3.1.1) were papain digested as described earlier (3.1.24) and the Fab fragments were stored at at ^o-20 C.

Immunoabsorbent purified IgG anti-BSA was prepared as described in 4.1.8.

5.1.13 RADIOLABELLING OF BSA

125

[I]-BSA was prepared as described in 2.14.

5.1.14 EFFECT OF EXOGENOUS GP60 ON IC-MEDIATED COMPLEMENT ACTIVATION

BSA-anti-BSA IC were formed at equivalence, four-times antibody excess and four-times antigen excess in normal serum at 37 C to which increasing amounts of gp60 had been added. The extent of complement activation was measured at different time intervals up to one hour. The reaction mixture contained 50 μ l normal serum, 50 μ l GVB⁺⁺, 10 μ l gp60 (0-15 μ g/10 μ l GVB⁺⁺) and 20 μ l immunoadsorbent purified IgG-anti-BSA (700 μ g/ml). At zero time sufficient BSA in 10 μ l VBS was added to achieve the desired antigen-antibody ratio.

Control tubes contained:

1. Serum with gp60 alone
2. Serum with BSA alone
3. Serum with anti-BSA alone
4. Complete reaction mixture containing 20 mM EDTA.

The volume of the reaction mixture was adjusted to 150 μ l with VBS, prior to its incubation at 37 C. At pre-determined time intervals, 25 μ l aliquots were removed and diluted in 100 μ l ice-cold VBS containing 20 mM EDTA, and after centrifugation (8000 g for 5 minutes in a Beckman Microfuge), the supernatants were stored at -70 C until tested for complement activation.

5.1.15 EFFECT OF ENDOGENOUS GP60 ON COMPLEMENT ACTIVATION

The role of the endogenous gp60 on complement activation was studied by examining the effect of Fab fragments of anti-gp60 on the PIP assay, total haemolytic complement activity and its effect on IC-mediated complement activation.

5.1.16 EFFECT OF Fab ANTI-GP60 ON PIP

125

[I]-BSA-anti-BSA IC were formed at equivalence in 12 normal and 12 rheumatoid arthritis sera using undiluted sera in the protocol described in 3.1.5. Purified gp60 was omitted from the reaction mixture, but Fab fragments of IgG anti-gp60 or normal rabbit IgG were added to each tube (140 μ g/ml final concentration). After incubation for one hour at 37 °C, 50 μ l of the reaction mixture was added to 1 ml ice-cold VBS containing 20 mM EDTA in a Microcap tube, which was then centrifuged (Beckman Microfuge for 5 minutes at 8000 g at room temperature).

An aliquot of the supernatant (250 μ l) was removed for counting soluble radioactivity. Controls included:

- 1) Total radioactivity input
- 2) Complete reaction mixture containing 20 mM EDTA.
- 3) Reaction mixture without Fab fragments.

The percentage of IC remaining soluble was calculated for each serum sample.

5.1.17 EFFECT OF Fab ANTI-GP60 ON INHIBITION OF PIP

The capacity of the same sera as those used in the PIP assay (5.1.16) to inhibit PIP was tested after heat-inactivation (56 C for 30 minutes) using the procedure described in 3.1.5. The assay was performed in the absence and presence of Fab fragments of IgG anti-gp60, or of normal rabbit IgG (140 µg/ml final concentration).

5.1.18 EFFECT OF Fab-ANTI GP60 ON TOTAL COMPLEMENT HAEMOLYTIC ACTIVITY

Sheep erythrocytes were coated with either IgG or IgM using the procedure described in 5.2.6.

Sera were diluted 1/30 in GVB⁺⁺, and increasing volumes of diluted serum (150 µl to 400 µl using 50 µl increments) were added to a triplicate series of test tubes. One hundred microlitres (100 µl) of GVB⁺⁺ containing Fab anti-gp60 or normal rabbit IgG Fab (each at a final concentration of 100 µg/ml in the reaction mixture) were added to the first two series of tubes. The volume of all three sets of tubes was adjusted to 500 µl by the addition of GVB⁺⁺ and then 300 µl EA (IgG) or EA (IgM) (5x10⁸ /ml GVB⁺⁺ were added to each tube. Control tubes included:

1. Cell blank - 500 µl GVB⁺⁺ + 300 µl EA
2. 100% lysis - same as cell blank but cells lysed in 2 ml water at the end of the experiment

The tubes were then incubated at 37 C for 30 minutes and the reaction was stopped by the addition of 2 ml isotonic saline. The tubes were centrifuged (2000 g for 5 minutes at 2 C) and the proportion of cells which had been

lysed was determined after reading the OD at 541 nm, using the following formula:

$$Y = \frac{\text{O.D test} - \text{OD cell blank}}{\text{OD 100 \% lysis} - \text{OD cell blank}}$$

CH50 levels were calculated by plotting Y/1-Y against serum dilution, and determining the serum dilution at which Y/1-Y equals one (i.e 50% lysis), using the formula :

$$\text{CH50 units/ml} = X \times \text{initial serum dilution.}$$

Where X = volume of diluted serum giving a value of Y/1-Y of one.

5.1.19 EFFECT OF Fab ANTI-GP60 ON IC-MEDIATED COMPLEMENT ACTIVATION

The same protocol described in 4.1.14 was used with the following exceptions.

- 1) Exogenous gp60 was omitted from the reaction mixture.
- 2) Fab fragments of IgG anti-gp60 or of normal rabbit IgG were added to the reaction mixtures at a final concentration of 140 µg/ml.

5.1.20 MEASUREMENT OF GP60 LEVELS IN NORMAL AND RHEUMATOID ARTHRITIS SERA

Serum levels of gp60 were determined using the ELISA protocol described in 3.1.16. Serum samples were diluted 1/100 in PBS-Tween.

RESULTS

5.2.1 EFFECT OF EXOGENOUS GP60 ON COMPLEMENT ACTIVATION

Gp60 produced dose- dependent inhibition of IC-mediated complement activation as measured by C1s-C1inhibitor complex formation (Fig. 5.1a;5.2), C4 and C2 consumption (Figs. 5.1c), C4a (Fig. 5.1b) and C3a (Fig 5.1d) generation, C4 and C2 consumption, (Fig 5.1c), and the formation of the C3-properdin complex (Fig 5.1e;5.3) and C5b-9 complexes (Fig 5.1f;5.4). The concentrations of the gp60 added to the reaction mixtures (12.5-100 ug/ml) correlated inversely with the concentrations of C1s-C1-inhibitor complex ($r = -0.997$), C4a ($r = -0.999$), C3a ($r = -0.958$), C3-properdin complex ($r = -0.967$), and C5b-9 complex ($r = -0.982$) generated, and with C4 ($r = -0.973$) and C2 ($r = -0.965$) consumption using IC formed at equivalence. Similar correlations were obtained when IC were formed in four-times antigen excess or four-times antibody excess (Table 5.1).

5.2.2 EFFECT OF ENDOGENOUS GP60 ON COMPLEMENT ACTIVATION

a) PIP

The mean PIP level in 12 normal sera was 86 (standard deviation $\pm 5\%$), ^{which} in the presence of Fab fragments of anti-gp60 rose to 96% (± 3.9) (Fig 5.5), while in the 12 rheumatoid arthritis sera the mean level was 50% (± 6.8), which a rose to 84% (± 9.6) upon the addition of Fab fragments of anti-gp60. Fab fragments of normal rabbit IgG had no effect on PIP when added to either normal or rheumatoid arthritis sera.

Figure 5.1:

- Effect of exogenous gp60 on IC-mediated complement activation. Gp60 (0-100 ug/ml) was added at time 0 of IC formation in normal serum.
- a) Effect on C1s-C1-inhibitor complexes formation
 - b) Effect of gp60 on C4a generation by IC
 - c) Effect of gp60 on C2 haemolytic consumption by IC
 - d) Effect of gp60 on C3a generation by IC
 - f) Effect of gp60 on the formation of C3/P complexes by IC
 - g) Effect of gp60 on the formation of C5b-9 complexes by IC.

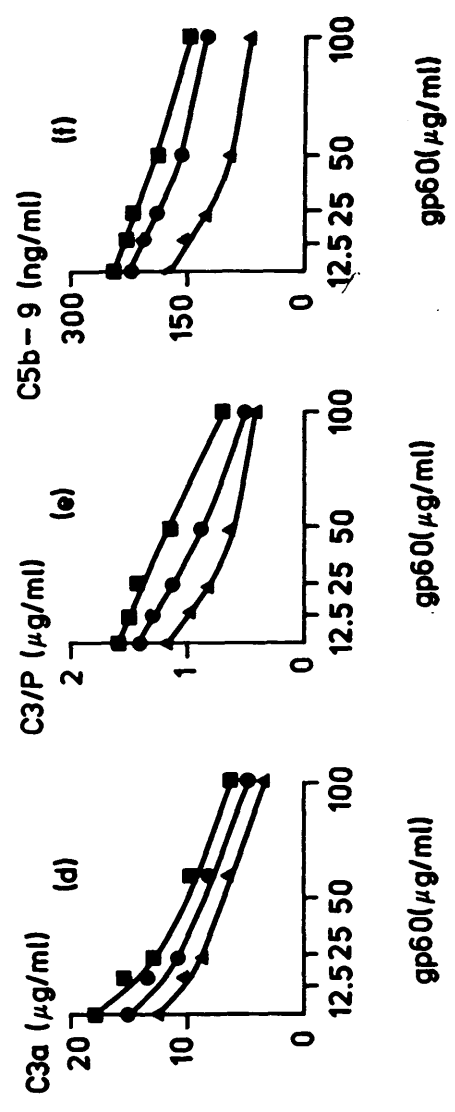
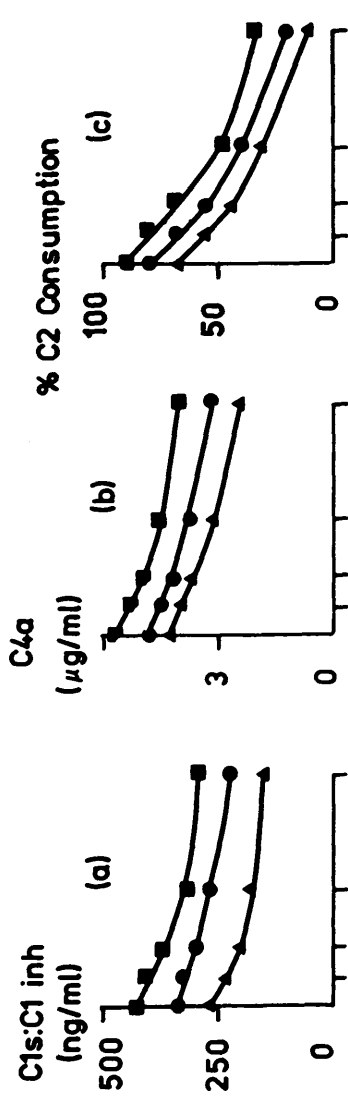


Figure 5.2:

Effect of gp60 preparations on C1 activation.

Gp60 (0-100 ug/ml) prepared by method 1 (▲—▲),
2 (●—●) or 3 (○—○) was added at time 0 of IC
formation in normal serum. The extent of C1
activation was measured by C1s-C1-inhibitor
ELISA.

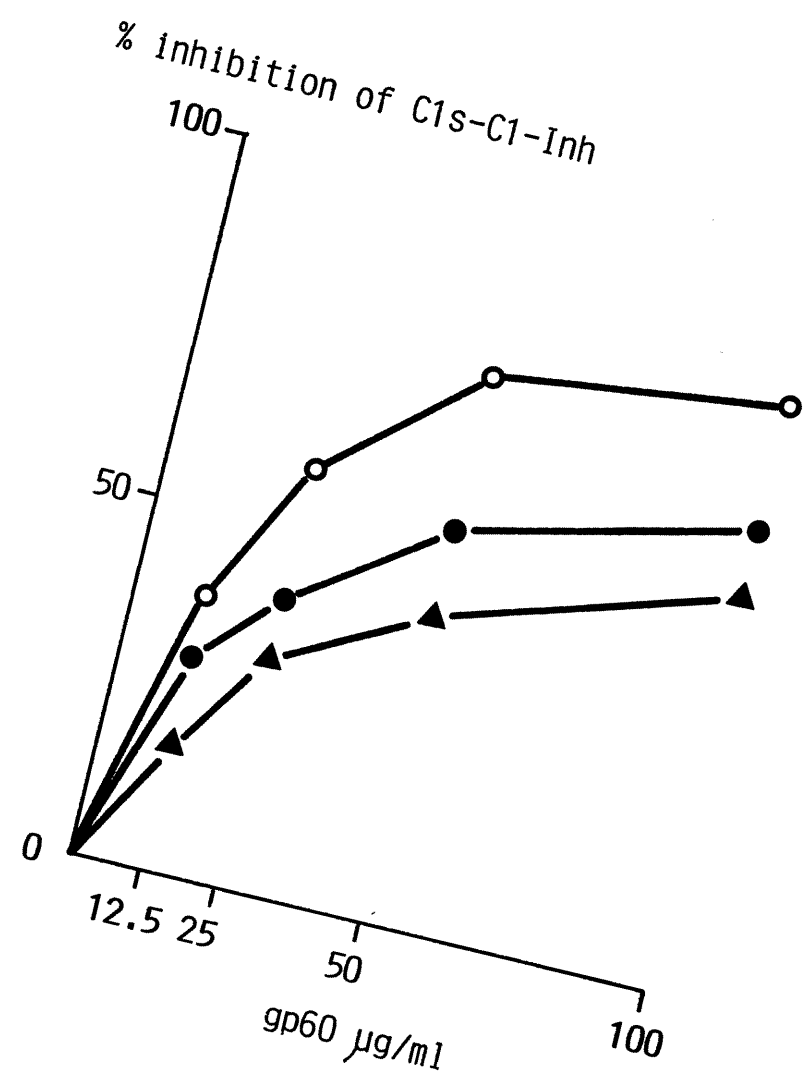


Figure 5.3:

Effect of gp60 preparation on IC-mediated formation of C3-Properdin complexes.

Gp60 (0-100 ug/ml) prepared by method 1 ($\Delta-\Delta$), prepared by method 2 ($\bullet-\bullet$) and gp60 prepared by method 3 ($\circ-\circ$) was added at time 0 of IC formation in normal serum and the formation of C3-Properdin complexes was measured by C3/P ELISA.

% inhibition of C3/P

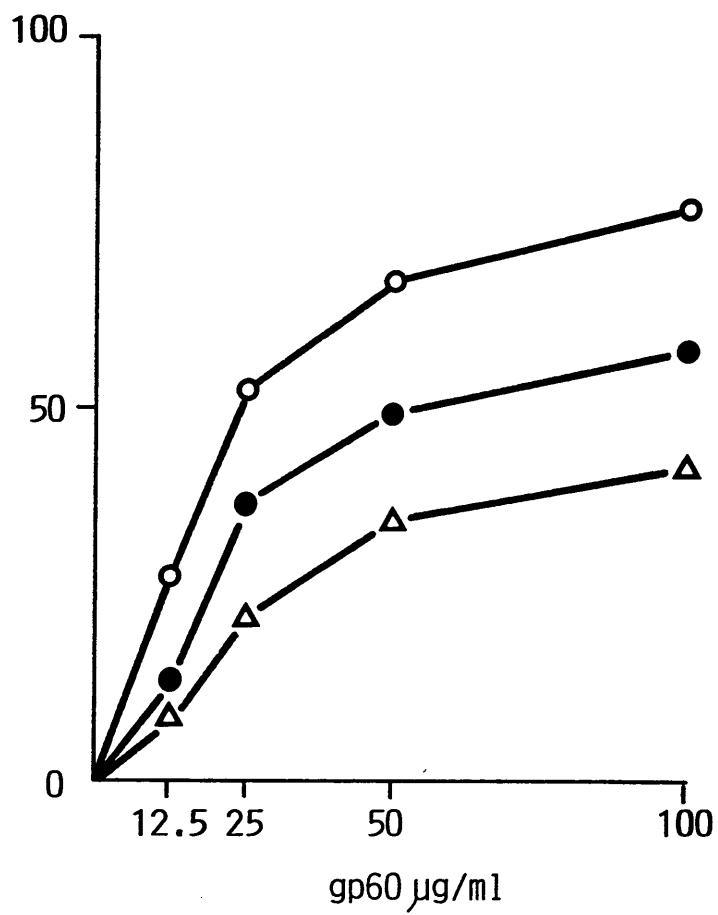


Figure 5.4:

Effect of gp60 preparations on C5b-9 complex formation by IC. Gp60 (0-100 ug/ml) prepared by method 1 (▲), 2 (●) or 3 (○) was added at time 0 of IC formation in normal serum. The extent of complement activation was measured by C5b-9 ELISA.

% inhibition of C5b-9

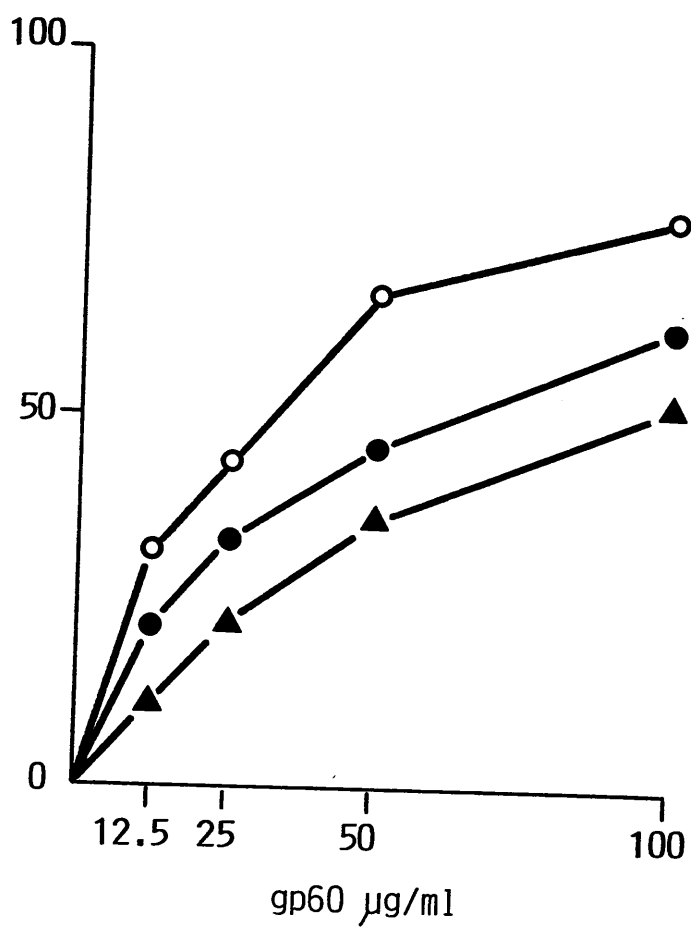


TABLE 5.1

Correlation of level of complement activation following activation of
by immune complexes in the presence of increasing concentrations of
gp60 (0 - 100 ug/ml)

Ag/Ab ratio	Correlation coefficient (r value)			
	C1s-C1-144	C4a	C3a	C2 C4 C3/P C5b-9
4 x Ag-excess	-0.991	-0.901	-0.961	-0.982 -0.946 -0.891 -0.955
Equivalence	-0.997	-0.999	-0.958	-0.973 -0.965 -0.996 -0.982
4 x Ab-excess	-0.964	-0.933	-0.949	-0.951 -0.998 -0.972 -0.969

In neither the normal nor the rheumatoid arthritis sera studied did the levels of PIP correlate with gp60 concentrations.

Heat-inactivated normal sera were able to inhibit PIP to a small extent with a mean value of 9.8% (± 3.8), while rheumatoid arthritis sera contained much higher levels of PIP inhibitory activity (mean 45% ± 21.4). In the presence of Fab fragments of anti-gp60, these values were reduced to 3.7% (± 2.7), and 28.5% (± 21.4) respectively (Fig 5.5b). The levels of PIP inhibitory activity in rheumatoid arthritis sera correlated with gp60 levels ($r=0.94$), but only a weak correlation was seen with the normal sera (Fig 5.6).

b) HAEMOLYTIC COMPLEMENT LEVELS

The mean CH50 level in normal sera was 168 units/ml (± 37) when measured with EA IgG and 137 (± 33) with EA IgM. In the presence of Fab fragments of anti-gp60, the former rose to 238 (± 58), while the latter was unchanged (Table 5.2). Similarly, the mean CH50 level determined with EA IgG in the rheumatoid arthritis sera was increased from 202 units/ml (± 77) to 307 (± 144) in the presence of Fab anti-gp60, while the EA IgM CH50 levels remained unchanged at 214 (± 115). Fab fragments of normal rabbit IgG had no effect on the CH50 assay.

c) IC-MEDIATED COMPLEMENT ACTIVATION

IC produced increased complement activation in sera t_0 which Fab anti-gp60 had been added as witnessed by the increased levels of C1s-C1 inhibitor, C3-properdin and C5b-

Figure 5.5:

Effect of endogenous gp60 on PIP and inhibition of PIP.

a) % PIP of 12 normal (\bigcirc) and 10 rheumatoid arthritis sera (Δ) before and after the addition of Fab fragments of anti-gp60 to a final concentration of 140 ug/ml.

b) % inhibition of PIP produced by 12 normal (\bigcirc), and 10 rheumatoid arthritis sera (Δ), before or after the addition of Fab fragments of anti-gp60 to a final concentration of 140 ug/ml in the reaction mixture.

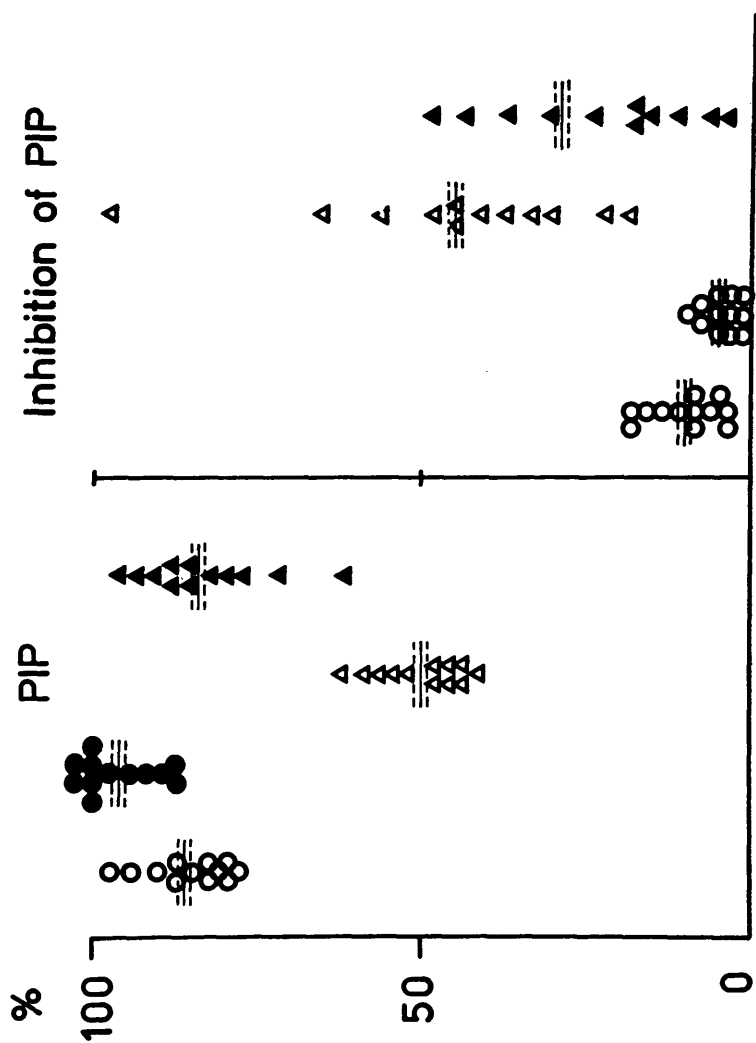


Figure 5.6:

Relationship between gp60 levels in normal (O)
and rheumatoid arthritis (▲) sera and their
ability to inhibit PIP.

% INHIBITION OF

PIP

100

$r=0.877$

$n=24$

50

0

83

166

gp60 ($\mu\text{g/ml}$)

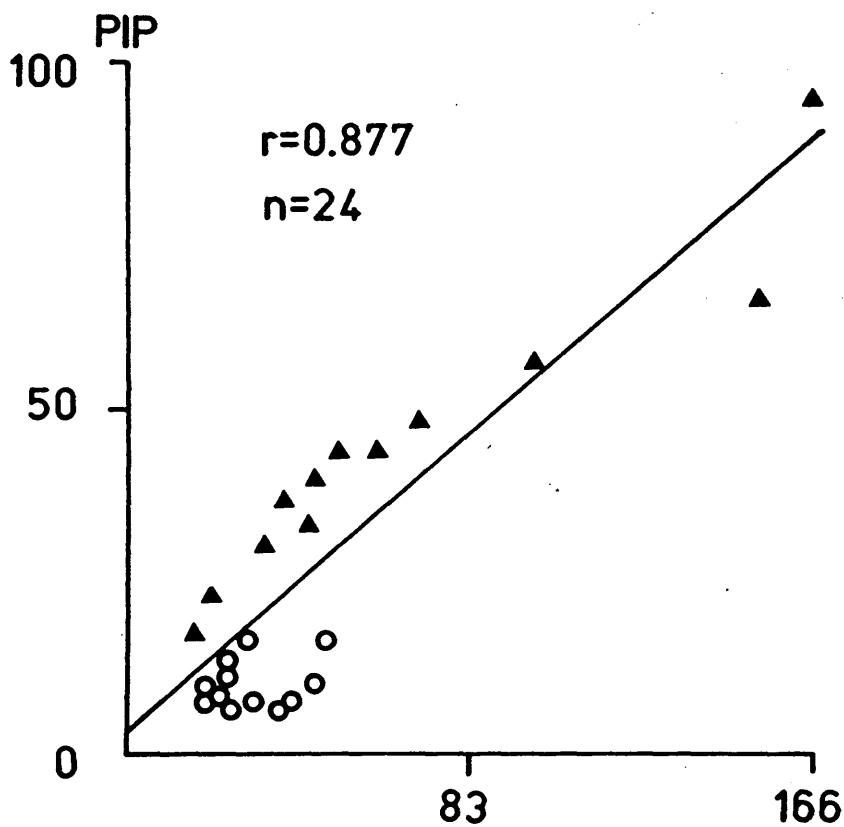


TABLE 5.2

Effect of Fab Anti-gp60 on haemolytic complement

NHS*		RAS**			
	Nil	Fab -gp60	NR.Fab***	Nil	Fab -gp60 NR Fab
EA (IgG)	168 ± 11.0****	238 ± 18	159 ± 18	202 ± 13	307 ± 19 200 ± 14
EA (IgM)	137 ± 10	137 ± 11	138 ± 11	214 ± 19	210 ± 16 212 ± 18

* Normal human serum (n = 10)

** Rheumatoid arthritis serum (n = 9)

*** Fab fragments prepared from normal rabbit IgG

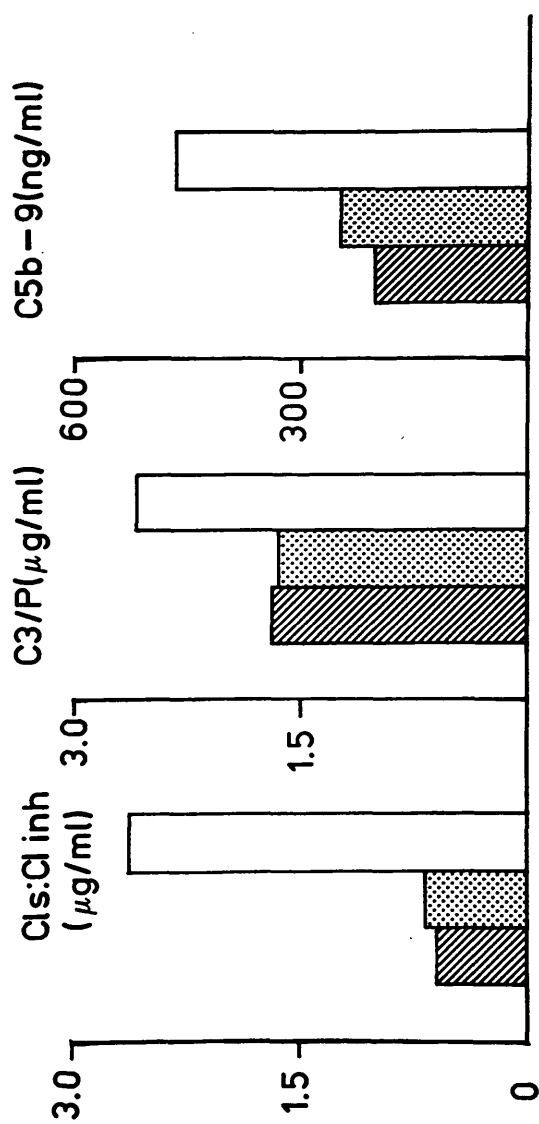
**** Units/ml (mean ± standard deviation)

9 complexes (Fig 5.7), while normal rabbit Fab fragments had no effect on IC-mediated complement activation (Fig. 5.7).

The addition of Fab fragments of anti-gp60 and of normal rabbit IgG to normal serum in the absence of IC did not produce complement activation.

Figure 5.7:

Effect of endogenous gp60 on IC-mediated complement activation. C1s-C1-inhibitor, C3-Propertdin and C5b-9 complex formation was measured after incubation of serum in absence (☒) or presence of Fab fragments of anti-gp60 (☐) or of normal rabbit IgG (☒). BSA-anti-BSA IC were formed in the serum at time 0 and the levels of activation products were measured after 60 minutes at 37 ° C.



DISCUSSION

The observation that the binding site for gp60 was localised on the Fc piece of IgG, suggested the possibility that gp60 might inhibit PIP by preventing the binding of C1 to IC with a reduction in classical pathway activation. The first step in the activation of the classical pathway is the binding of C1 to IgG or IgM by means of the C1q subcomponent (Reid & Porter, 1981). In the previous chapter it was shown that in a solid-phase system, gp60 competitively inhibited the binding of ¹²⁵[I]-C1q to IgG, but not to IgM. These data not only confirmed that the binding site for gp60 is only present on IgG, but also suggested that binding of gp60 to IgG could prevent the binding and activation of C1 by IgG-containing IC. This possibility was also suggested by the results of previous studies in which it was noted that 1) rheumatoid arthritis sera contained a factor which inhibited the binding of C1q to IC, and that serum levels of this activity correlated with their ability to inhibit PIP (Niven & Whaley, 1986), 2) Levels of PIP inhibitory activity in rheumatoid arthritis sera correlated inversely with the extent of C4 turnover (Naama et al, 1983).

The effects of exogenous gp60 on complement activation were studied in a fluid-phase system in which IC were formed in normal human serum. Gp60 produced dose-dependent inhibition of C1 activation as shown by decreased formation of C1s-C1-inhibitor, which in turn was associated with reduced activation of C4 and C2 as shown by reduced generation of C4a and reduced C4 and C2 consumption. Thus

formation of the classical pathway C3 convertase (C4b2a) would have been reduced by gp60, which could account for the dose-dependent inhibition of C3 activation, as shown by reduced C3a generation. As a result of the reduction in C3 activation, less C3b would be available to bind to IC. As the covalent binding of C3b to IC is the essential step in PIP (Naama et al, 1985) the inhibition of PIP produced by gp60 can be readily explained.

The mean concentration of gp60 in 10 normal sera was $34 \mu\text{g/ml}$ (± 5.2), while increased levels were found in 9 RA sera (mean $97 \mu\text{g/ml} \pm 43$).

The presence of gp60 in normal serum suggests that it may regulate complement activation in-vivo. Evidence for such a regulatory role was obtained from three sets of experiments. In the first it was found PIP was increased and inhibition of PIP was almost abolished when Fab fragments of anti-gp60 were added to normal sera. However, in rheumatoid arthritis sera, although Fab fragments of anti-gp60 increased the levels of PIP, they did not return to normal, and levels of PIP inhibitory activity were only reduced by 40-50%. It is possible that the amount of Fab anti-gp60 ($140 \mu\text{g/ml}$) added to these rheumatoid arthritis sera may have been insufficient to neutralise all the gp60 activity, particularly as levels were markedly elevated in some. This is unlikely to be the case as the same amount of Fab anti-gp60 was able to neutralise the inhibitory activity of $12.5 \mu\text{g}$ of purified gp60 in a PIP assay. Thus the failure to neutralise all the inhibitory activity in rheumatoid arthritis sera with Fab anti-gp60 argues that gp60 may not be the only factor involved in inhibition of

PIP in these sera. Indeed, Mitchell et al, 1984; Balastrieri et al, 1984 and O'Sullivan et al, 1988 have shown that purified IgM-RF will also inhibit PIP. In addition partially solubilised IC are also thought to play a similar role in systemic lupus erythematosus sera (Baatrup et al, 1983).

The relative roles of gp60, IgM-RF and possibly other factors in the inhibition of PIP require further study. However, the close correlation of serum levels of gp60 and the ability of rheumatoid arthritis sera to inhibit PIP supports the notion that gp60 plays a role in this activity.

In contrast to the situation in rheumatoid arthritis sera, the observation that Fab anti-gp60 almost completely abrogated the ability of normal sera to produce limited inhibition of PIP suggests that gp60 is entirely responsible for these low levels of inhibitory activity.

In the second series of experiments it was shown that the addition of Fab anti-gp60 to normal and rheumatoid arthritis sera increased the levels of total haemolytic complement activity when EA IgG were used. In contrast no effect was seen when EA IgM were used as the target cells. These results also confirm the specificity of gp60 to IgG. Finally, when Fab anti-gp60 was added to normal serum, IC produced increased complement activation.

Taken together these findings demonstrate that gp60 plays a regulatory role in the activation of the classical pathway by IgG- containing IC. This regulatory role differs from that of C1-inhibitor as neutralisation of gp60

activity by Fab anti-gp60 did not result in spontaneous C1 activation which is observed when C1-inhibitor activity is neutralised in this way (Gronski et al., 1986). The biological significance of this regulatory role of gp60 on complement activation remains unclear, and further investigations are required.

CHAPTER SIX

FINAL DISCUSSION

The formation of, or the deposition of antigen-antibody complexes (IC) in the tissues is considered to be an important mechanism of tissue injury (Theofilopouls & Dixon, 1979).

It is well known that when IC are formed around equivalence or in antibody-excess in buffer, they tend to precipitate. However, IC formed in the presence of normal serum remain small and soluble. The ability of normal serum to prevent immune precipitation is mediated by the complement system (Schifferli et al, 1980). The use of purified complement components has shown that the classical pathway of complement is responsible for this activity (Naama et al, 1984), while the alternative pathway serves simply to provide a backup support system (Naama et al, 1984; 1985). The final and critical step in complement-mediated prevention of immune precipitation (PIP), appears to be the binding of C3b to IC to prevent the formation of large insoluble immune precipitates (Naama et al, 1985).

The sera of patients with rheumatoid arthritis who are sero-positive for IgM-RF are unable to retain IC in solution because they contain an inhibitor of PIP (Naama, Mitchell & Whaley, 1983). During attempts to purify the PIP-inhibitory material from rheumatoid arthritis sera using IgG-Sepharose affinity chromatography followed by gel filtration chromatography, the PIP-inhibitory activity co-eluted with IgM-RF (Mitchell et al., 1984). However, IgM-RF levels did not correlate with the PIP-inhibitory activity present in these sera, which suggested that IgM-RF could, at the most, be only partly responsible for their PIP-

inhibitory activity. As normal human serum was shown to contain a low level of PIP-inhibitory activity, it was suggested that inhibition of PIP might be due to a plasma protein which was present in normal serum and that increased levels were found in rheumatoid arthritis sera.

The main object of these studies was to purify and characterise this PIP-inhibitor, and define its mode of action on the complement system.

The first objective was achieved as described in detail in chapter three. The protein isolated had a molecular weight of 60 kD on SDS-PAGE run under reducing and non-reducing conditions, and because it stained positively in the PAS reaction it was called glycoprotein 60 (gp60).

When prepared by method one, gp60 had sedimentation and gel filtration characteristics which were identical with a minor fraction of the gp60 which was present in whole serum, in which the characteristics of the majority of the gp60 were similar to those of IgM (19S sedimentation coefficient; 1000 kD on gel filtration). When gp60 was isolated in the presence of protease inhibitors, the purified protein shared the same sedimentation and gel filtration characteristics as the larger molecular weight component in whole serum.

As both the high molecular weight and low molecular weight forms of gp60 appears as a single polypeptide chain on SDS-PAGE run under either reducing or non-reducing conditions it is probable that the high molecular weight gp60 is a polymer of the low molecular weight form, which

probably represents the monomer. The number of monomeric subunits which make up the polymer, assuming molecular weights of 60 kD and 1000 kD for the monomer and polymer respectively, must be 16-17 which is extremely high. For instance, the C3b receptor (CR1) has a molecular weight of 200 kD on SDS-PAGE but on gel filtration chromatography it is eluted with an apparent molecular weight of 1.2×10^6 (Fearon, 1979), which means that 6 subunits make up the polymer. Likewise, C4BP is made up of 6 identical subunits (Dhalback & Stenflo, 1981). It is possible that the estimate of 1000 kD for the molecular weight of the polymer is inaccurate. It could also be argued that the high molecular weight of gp60 in serum could be due to the complexing of gp60 with another serum constituent, such as IgG. The observation that purified gp60 (purified by methods 2 and 3) had a similar molecular weight to serum gp60 suggests that the second explanation is not the case. Although the polymeric form of gp60 sedimented along with IgM on sucrose density gradient centrifugation, and co-eluted with IgM on gel filtration chromatography, C4BP (molecular weight 590 kD) and fibronectin (molecular weight 440 kD) also sediment close to IgM on sucrose density gradient centrifugation and co-elute with IgM on Sephacryl S-300 gel filtration chromatography. Thus the molecular weight of polymeric gp60 may be considerably less than 1000 kD, which would mean that the number of monomeric subunits required to form the polymer would be less than the previous estimate.

The observation that gp60 polymer migrated at the B γ globulin boundary on immunoelectrophoresis, while C4BP

migrates as a B¹-globulin suggests that polymeric gp60 has a higher molecular weight than C4BP. The low isoelectric point of gp60 (5.1-5.3) and the prealbumin mobility of the monomer suggest that the differences in electrophoretic mobility between gp60 and C4BP are not due to charge differences, unless the isoelectric point of C4BP is lower than that of gp60. There do not appear to be any data published on the isoelectric point of C4BP.

From the above discussion it is clear that the molecular relationship between gp60 polymer and gp60 monomer is a problem which requires further investigation. Approaches which could be used to solve this problem are electronmicroscopy, x-ray crystallography or neutron scattering studies, all of which may yield valuable information about the size, shape and subunit structure of plasma proteins. In the past they have been used successfully to study the structure of complement proteins. The molecular weight of the polymer could be determined by studying various physical properties such as the osmotic pressure of gp60 in solution or the ability of gp60 to lower the freezing point of water.

Although gp60 (prepared by all the methods) was relatively resistant to proteolysis, the conversion of polymer to monomer when protease inhibitors were not included in the purification procedure was reproducible. Thus it appears that limited proteolysis of the polymer by a plasma/ serum protease accounts for the transformation from the polymeric to the monomeric form. The positive staining of gp60 in the PAS reaction and the

sensitivity of the molecule to proteases, showed that gp60 was a glycoprotein.

The true molecular weight of gp60, as determined by the Ferguson technique (Ferguson, 1964), was 43 kD which is in rough agreement with the results of chemical deglycosylation with trifluoromethylsulphonic anhydride. Thus approximately 28% of the gp60 molecule consists of carbohydrate. As neuraminidase treatment reduced the molecular weight of gp60 to 52 kD, it is probable that approximately 40% of the carbohydrate on gp60 is sialic acid. The relative high carbohydrate content of gp60 is not unusual for a plasma protein; approximately 30% of the molecular weight of C1-inhibitor consists of carbohydrate (Harrison, 1983). The function of the carbohydrate constituents of glycoproteins is generally unknown. There is evidence that they may be involved in protein catabolism (Hughes, 1976). However, if one removes sialic acid from the glycoproteins of the complement system there is no loss of functional activity. Thus, although studies of the possible functional role of the carbohydrate component of gp60 should be undertaken, they ^{are} unlikely to yield much useful information.

Gp60 was purified from normal serum on the basis of its ability to inhibit PIP. The purified protein was shown to inhibit both PIP and solubilisation, although it was more effective at inhibiting PIP. This is probably related to its ability to bind to the Fc piece of IgG and inhibit activation of the classical pathway, which is essential for PIP but non-essential for solubilisation. Gp60 was more effective at inhibiting PIP and

solubilisation when IC were formed at equivalence or in antibody-excess, compared with those formed in antigen-excess. The explanation for this observation is not immediately apparent, but could be related to the intrinsic solubility of IC formed in antigen-excess, or could be due to a failure of gp60 to bind effectively to fluid-phase IC formed in antigen-excess. Certainly complement activation is achieved more efficiently by IC formed in antibody-excess or at equivalence, than by those formed in antigen excess (Osler & Heidelberger, 1948). Thus if the binding of C1 is less efficient in antigen-excess and as I have shown that gp60 and C1 compete with each other in terms of binding to IgG, it could be expected that gp60 will bind less effectively to IC formed in antigen-excess. Future studies into the mechanism of inhibition of PIP by gp60 should investigate the binding of gp60 to soluble IC formed at different antigen-antibody ratios.

The solid-phase studies showed that gp60 bound to IgG but not to the IgM or IgA isotypes. Using defined fragments of IgG, the binding site for gp60 was shown to be located on the Fc piece. This conclusion was supported by assays which showed that other Fc binding proteins could competitively inhibit the binding of gp60 to IgG. The precise binding site for gp60 on IgG has yet to be determined. The data presented in this thesis show that it could involve either the CH2 or CH3 domain, or both. The binding site for C1q is located within the CH2 domain (Colomb & Porter, 1975), while the epitopes recognised by RF may be located on either the CH2 or CH3 domains

(Jefferis, 1980), and Staphylococcus protein A binds to both the CH2 and CH3 domains (Deisenhofer, 1981).

Experiments performed by Dr. G.P. Sandilands of this department show that EA(IgG) incubated with gp60 do not bind to the Fc receptors of lymphocytes monocytes or polymorphonuclear leukocytes, which recognise either the CH2 or CH3 domains of IgG.

C1q, gp60, RFs and Staphylococcus protein A are large molecules, so their binding to one site on the Fc piece of IgG might be expected to interfere with the binding of other proteins to the IgG Fc piece, despite the fact that they may not share the same binding site. More accurate localisation of the gp60 binding site on the IgG Fc piece could be achieved by studying the effect of synthetic peptides, which correspond to different regions of the Fc piece, on the binding of gp60 to IgG (Lukas, Munoz & Erickson, 1981). Alternatively, the effect of chemical modification of amino acid residues in the Fc piece on the binding to gp60 could be studied.

As the molar concentration of IgG in whole serum is high (6.7×10^{-6} mol/l), and as it does not appear that gp60 circulates bound to IgG, one must conclude that gp60 can only bind to IgG once it has become aggregated in the form of an IC. The problem underlying the recognition by gp60 of IgG aggregated in IC must be similar to those facing C1q or Fc receptors, which have been the subject of great debate (reviewed by Burton, 1985). The discussion of the mechanism of recognition of aggregated IgG cannot be continued without reference to the IgG subclass specificity of gp60 binding. As the primary amino acid structures of

the CH2 and CH3 domains of the four different IgG subclasses are remarkably constant, it is unlikely that differences in the amino acid sequences account for the subclass differences in the binding of gp60, C1q and RFs to IgG. The possibility that hidden binding sites are exposed in the IgG heavy chain following antigen-binding has been suggested, but available data do not support this concept (reviewed by Burton, 1985). However, in the case of C1q, there is no doubt that it binds extremely efficiently to IgG antibody on the surface of erythrocytes (Isenman, Dorington & Painter, 1975). If the binding site was available on monomeric IgG, there would be sufficient fluid-phase monomeric IgG available to prevent any C1q binding. Thus a conformational change in IgG antibody must occur following its binding to antigen. The hinge region plays a key role in this process. The hinge regions of IgG1 and particularly IgG3 are large and allow the Fab arms great flexibility so that on binding to antigen, the binding domains for C1q and Fc receptors on the Fc piece became accessible. This has been used to explain why the IgG1 and IgG3 subclasses activate C1 and bind to Fc receptors far more effectively than IgG2 and IgG4 which have much smaller and less flexible hinge regions (Burton, 1985).

The recognition of aggregated IgG, and the subclass specificity of the binding of gp60 can probably ^{be} explained in the same way. It would be of interest to study the binding of gp60 to IgG molecules which have deletions in their hinge regions (Dorrington & Klein, 1982). These

molecules bind to C1q and Fc receptors far less effectively than normal IgG molecules.

The studies of the role of gp60 in complement activation showed that the addition of purified gp60 to whole serum reduced complement activation by IC. This observation raised the possibility that gp60 already present in serum might regulate the activation of complement. This possibility was investigated by neutralisation of serum gp60 activity by the addition of Fab anti-gp60 to the serum. Using this procedure it was shown that neutralisation of serum gp60 activity resulted in 1) increased levels of haemolytic complement using EA(IgG) targets, 2) increased level of complement activation products following the formation of IC in whole serum, and 3) increased levels of PIP activity in normal serum.

These findings show that serum gp60 does regulate complement activation, although the biological importance of this role is by no means clear.

When IC are formed in-vivo they will contain circulating antibodies of all isotypes. Thus, although gp60 binds to IgG and inhibits complement activation, it will not prevent complement activation by IgM antibody. Thus, how gp60 affects complement activation by IC containing both IgG and IgM antibodies requires investigation, as does the fate of such IC following complement activation. The availability of murine anti-DNP monoclonal antibodies of different isotypes (Johnson et al, 1988) will allow these problems to be investigated.

The failure of Fab anti-gp60 to neutralise completely all the PIP-inhibitory activity in rheumatoid arthritis

sera suggests that gp60 may not be the only constituent of these sera which inhibits PIP. Earlier studies (Mitchell et al, 1984; Balastrieri et al, 1984; O'Sullivan et al, 1988) showed that IgM-RF did inhibit PIP. Although most IgM-RF preparations contain variable amounts of gp60, it is possible that IgM-RF does inhibit PIP. The high correlation between inhibition of PIP and the gp60 concentrations in rheumatoid arthritis sera which was found in this study, stands in contrast to the poor correlation which was found between inhibition of PIP and IgM-RF levels found in a previous study (Webb et al, 1986). Thus the relative importance of gp60 and IgM-RF in the inhibition of PIP requires further study.

The final aspects of gp60 which requires discussion is the identity of the molecule. Using serological techniques it has been shown that gp60 has not been recognised previously as a plasma protein. This may seem difficult to believe as the concentration of gp60 in normal serum is approximately 30 ug/ml. However, on account of this relatively low concentration, together with its electrophoretic mobility (gp60 migrates in the $\beta_2 - \gamma$ boundary zone along with a large number of other plasma proteins, some of which are present in far higher concentrations) may account for the failure to detect gp60 previously. It should also be pointed out that it is difficult to detect C2, a protein of similar serum concentration on immunoelectrophoresis of whole serum using anti whole serum as the developing antibody. C2 was detected on the basis of its biological activity.

The observation that gp60 is an Fc binding protein and that the binding site is expressed preferentially on the IgG1 and IgG3 subclasses indicates that gp60 has characteristics in common with leucocyte Fc receptors (Burton, 1985; Anderson & Looney, 1986). There appear to be at least three distinct classes of human leucocytes Fc receptors, FCRI, FCRII, and a low-affinity receptor termed FCRlo (Anderson & Looney, 1986). This classification is based on immuno-reactivity with defined monoclonal antibodies, mobility on SDS-PAGE and distribution on different populations of leucocytes. The molecular weight of FCRlo-70 kD (Anderson & Looney, 1986), is closest to that of gp60. It has been shown previously that normal serum contains a factor which is able to reconstitute Fc receptor activity on peripheral blood lymphocytes, which during incubation with IC have lost their surface Fc receptors (Reid et al, 1983; Sandilands, Peel & MacSween, 1984). Fc receptors have also been detected in normal mouse serum (Khyat et al, 1984; Pure et al, 1984). The observation that the fluid-phase murine lymphocyte Fc receptor inhibits complement activation by the classical pathway (Fridman, Nelson & Liabeuf, 1974; Molenaar et al, 1977), also supports the notion that gp60 may be an Fc receptor.

The next step in solving many of the outstanding problems of gp60 is to determine its amino acid sequence. Sequence data could then be analysed by computer and homologies with other proteins identified. Attempts at determining the amino acid sequence of gp60 have so far been unsuccessful. The protein appears to be relatively resistant to proteolysis. In the absence of amino acid

sequence, screening of cDNA libraries for cDNA clones coding for gp60 must depend on the use of expression libraries. Such studies are presently underway. Hopefully they will help shed some light on the biological role of gp60 in normal individuals and its role in the pathogenesis of immune complex disease.

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